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TITLE: Sensitization of Prostate Cancer Cells to Androgen Deprivation and Radiation  
Via Manipulation of the MDM2 Pathway

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14. ABSTRACT  MDM2 is a feedback regulator of p53. We found that MDM2 is central to the apoptotic response of prostate cancer cells to radiotherapy (RT), androgen deprivation (AD) and RT+AD. In vitro measurements in androgen sensitive and insensitive cells revealed that antisense-MDM2 (AS-MDM2) significantly enhanced apoptosis and overall cell death (clonogen survival) in response to all of these treatments. An orthotopic model using wild-type LNCaP cells injected into the prostates of nude mice corroborated the in vitro findings, particularly in terms of sensitization to AD. The mouse model involved determinations of growth inhibition through measurements of serum PSA and MRI-based tumor volume. Treatment with AS-MDM2+AD and AS-MDM2+AD+RT resulted in the greatest growth inhibition, compared to the other groups. We have also measured MDM2 expression using immunohistochemistry in men treated on Radiation Therapy Oncology Group trials 86-10 and 92-02. MDM2 overexpression was associated with a higher rate of distant metastasis and mortality, independent of conventional factors, treatment, Ki-67 and p53. We now have a method not only for identifying men at high risk of treatment failure, but also for selecting men who would have the greatest potential benefit from therapeutically targeting MDM2. Men in all prostate cancer risk groups should benefit.					
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## INTRODUCTION

Androgen deprivation (AD) and radiation (RT) are two of the most common methods of treating prostate cancer, yet for men with high risk features treated with AD+RT and those with more advanced disease treated with AD alone the failure rates at 5 years are over 40%. An ideal new therapy would be one that could be used to improve the results of men at all stages of their disease. One way of accomplishing this is to enhance the response of prostate cancer cells to AD. In the normal prostate, androgen deprivation results in the induction of apoptosis in over 80% of the epithelial cells within 10 days. In contrast, the apoptotic response of prostate tumor cells occurs in the minority. Rather than the induction of cell death, there is a major shift into a resting state. In order to take full advantage of AD as a therapeutic modality, the mechanisms responsible for the diminished apoptotic response of tumor cells must be understood and overcome. The findings that have come out of this grant demonstrate that MDM2 modulates the response of both androgen sensitive<sup>1</sup> and insensitive prostate (abstract submitted for a meeting presentation; paper in preparation) cancer cells to AD. The latter finding is surprising in that even prostate cancer cells that are no longer growth inhibited by AD, respond to the suppression of MDM2 by antisense-MDM2 (AS-MDM2).

The other component of the strategy described here, is to promote increased cell death in response to RT. Local control is essential to preventing local progression and metastasis. As we have demonstrated as a consequence of this grant, biochemical failure after radiotherapy is a determinant of distant metastasis<sup>2</sup>, and radiation dose escalation reduces the rate of both biochemical failure<sup>3</sup> and distant metastasis<sup>4</sup>. MDM2 suppression also enhances the response of prostate cancer cells to RT<sup>5</sup>, which should have the same effect as further escalating RT dose.

The primary objective of the research was to enhance the response of prostate cancer cells through the manipulation of MDM2 suppression. We also investigated the potential of radiosensitization by E2F1 overexpression through and adenoviral vector.<sup>6</sup> Both MDM2 and E2F1 are involved in the regulation of apoptosis through common and independent pathways. The investigation of the interaction of E2F1 overexpression and MDM2 suppression is the subject of a new DOD proposal that developed as an offshoot to this current grant.

## BODY

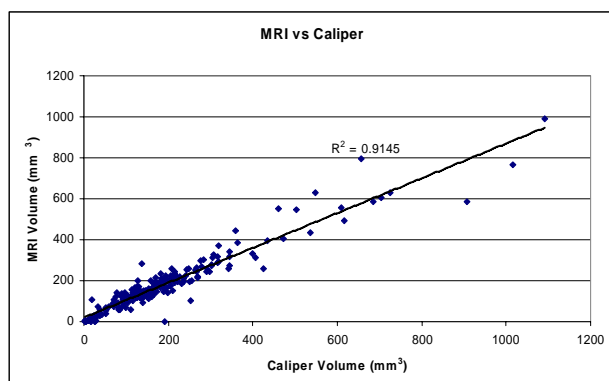
*Task 1.* Determine the impact of MDM2 suppression and overexpression on the interaction of AD and RT in promoting cell death and inhibiting prostate cancer growth in vitro and in vivo.

- a.* Complete in vitro apoptosis measurements on LNCaP cells treated in vitro with AS in combination with AD, RT, and AD+RT. Months 1-6.
- b.* Baseline cell viability, cell number apoptosis, and clonogenic assays of LNCaP-MST. Months 1-6.
- c.* Time course experiments of AS effects on AD, RT, and AD+RT in LNCaP and LNCaP-MST cells. Months 7-12.
- d.* In vivo experiments of the action of AS on LNCaP and LNCaP-MST cells. Months 7-36

We have completed and published the components *a-c* in Task 1; the results have been presented at two meetings<sup>7,8</sup> and published in two papers.<sup>1,5</sup> The in vivo experiments (component *d*) have taken longer than expected, but, are now complete. The results shown in **Table 1** summarize the effects of AS-MDM2 with AD, RT and AD + RT. Titration experiments were performed for AS-MDM2, first testing 12.5 mg/kg/day for 10 days. We saw minimal effect at

this level. In the experiment shown, AS-MDM2 was used at 25 mg/kg/day for 15 days over three weeks. Radiation dose was also titrated and 5 Gy resulted in a minimal response when administered alone, as compared to a significant response at 7.5 Gy. Thus, 5 Gy was felt to be optimal for the demonstration of radiosensitization. AD, when used, was initiated 3 days prior to the start of AS-MDM2. A unique aspect of these in vivo studies is that progression was assessed

**Figure 1. MRI-Based Tumor Volume Versus Caliper-Based Tumor Volume**



by measuring freedom from tumor volume failure (FFTVF) using a small animal MRI to quantify tumor growth and by measuring freedom from biochemical failure (FFBF) based on serum PSA changes.

**Figure 1** shows that there was a strong correlation between MRI-based tumor volume and direct tumor volume approximation using caliper measurements on prostate tumors removed surgically (the tumors were all grown orthotopically in the prostates of nude mice). A similar relationship was

observed for serum PSA versus caliper-based tumor volume, although the MRI-based tumor volume measurements were more robust and less affected by the use of AD.

The results in **Table 1** are in general agreement with the in vitro findings that AS-MDM2 sensitizes LNCaP cells to AD and RT.<sup>1,5</sup> The major treatment effect in vivo was seen with the combination of AS-MDM2 + AD. The improvement in FFBF and FFTVF was significantly greater than for MM + AD. There was a trend for further improvement using AS-MDM2 + AD +

RT. Our data show that AS-MDM2 sensitizes cells in vitro and in vivo to AD ± RT and should, therefore, have an impact on prostate cancer patients with the full spectrum of disease.

There are two additional lines of research that developed over the grant funding period. In one, E2F1 overexpression through the use adenoviral-E2F1 (Ad-E2F1) was found to be a potent radiosensitizer of wild-type LNCaP (androgen sensitive, p53<sup>wild-type</sup>) and PC3 (androgen insensitive, p53<sup>null</sup>) cells.<sup>6</sup> While, E2F1 overexpression has been shown previously to sensitize

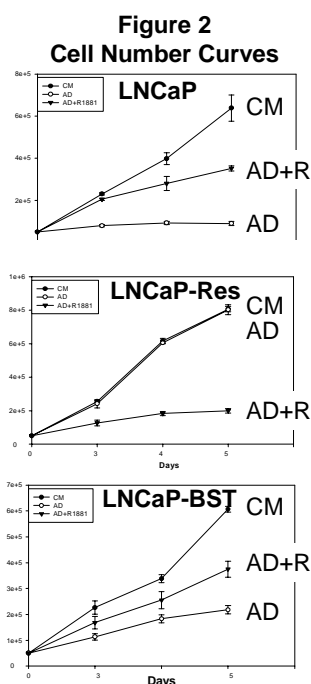
**Table 1. Freedom From Biochemical and MRI-Based Tumor Volume Failure in LNCaP Cells Grown Orthotopically in the Prostates of Nude Mice**

Group	FFBF 6 wks	FFBF 10 wks	FFTVF 10 wks	Combined 10 wks
No Tx	0% (0/9)	0% (0/9)	0% (0/9)	0% (0/9)
MM	8% (1/12)	8% (1/12)	16% (2/12)	8% (1/12)
AS-MDM2	10% (1/10)	10% (1/10)	30% (3/10)	10% (1/10)
MM+RT	18% (2/11)	0% (0/11)	18% (2/11)	0% (0/11)
MM+AD	8% (1/12)	25% (3/12)	25% (3/12)	16% (2/12)
MM+AD+RT	38% (5/13)	46% (6/13)	38% (5/13)	38% (5/13)
AS+RT	10% (1/10)	0% (0/10)	30% (3/10)	0% (0/10)
AS+AD	62% (8/13)	54% (7/13)	62% (8/13)	54% (7/13)
AS+AD+RT	77% (10/13)	69% (9/13)	77% (10/13)	69% (9/13)

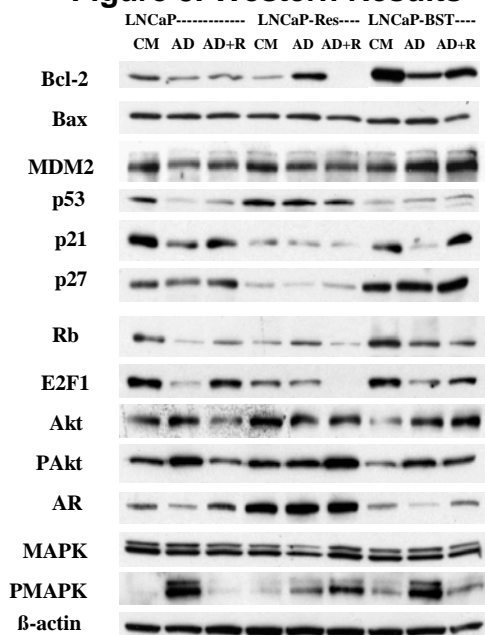
No Tx= no treatment; MM= mismatch control at 25 mg/kg injected intraperitoneally for 5 days/week for 3 weeks; AD= androgen deprivation via orchiectomy started 3 days prior to AS-MDM2; AS= AS-MDM2 at 25 mg/kg injected intraperitoneally for 5 days/week for 3 weeks; RT= 5 Gy pelvic radiation therapy given after 5 days of AS treatment; FFBF= freedom from a PSA of >1.5 ng/mL at 6 or 10 weeks from treatment start; FFTVF= freedom

tumor cells to RT, this is the first study to demonstrate this effect in prostate tumor cells and the first to use an adenoviral vector in combination with RT. The other line of research involved the action of AS-MDM2 on prostate cancer cells that had become resistant to AD-mediated growth

inhibition. An AD growth-resistant LNCaP cell line (LNCaP-Res) was developed by growing the cells in AD medium for greater than one year.



**Figure 3. Western Results**



**Table 2 . Effects of AS-MDM2 on Caspase-3+7 Activity in LNCaP-Res and LNCaP-BST cells**

Treatment	Caspase-3 + 7 activity (RFLU)			
	LNCaP-Res		LNCaP-BST	
	M ± SEM	p*	M ± SEM	p*
CM+LC	212 ± 27	----	218 ± 16	----
CM+MM-MDM2	285 ± 35	1.000	236 ± 19	1.000
CM+AS-MDM2	512 ± 22	0.469	337 ± 17	0.007
AD+LC	162 ± 25	0.01	124 ± 8	<0.0001
AD+MM	391 ± 97	0.450	200 ± 17	0.124
AD+AS*	871 ± 138	<0.0001	472 ± 32	<0.0001
AD+R1881+LC	204 ± 24	<0.0001	258 ± 6	<0.0001
AD+R1881+MM-MDM2	243 ± 27	1.000	287 ± 7	1.000
AD+R1881+AS-MDM2	337 ± 34	1.000	399 ± 13	0.002

LNCaP-Res and LNCaP-BST cells were treated with AS-MDM2 (200nM) alone or in combination with AD ± R1881. Caspase 3+7 activity was measured by fluorometric assay.

**Abbreviations:** LC = lipofectin control; AS = antisense MDM2; MM = antisense mismatch.

\*Compared to group above, One way Anova, Bonferroni test. The data shown represent the average values (± SEM) from three independent experiments.

\* \*Other LNCaP-Res comparisons (n = 9 treatment groups): AD-AS versus CM-AS (p<0.0001). Other LNCaP-BST comparisons (n = 9 treatment groups): AD-AS versus CM-AS (p<0.0001).

appear to be similar to the reports of others and are noteworthy. In response to AD, bcl-2, Rb, and phosphoMAPK are upregulated and MDM2 downregulated. In contrast, in wild-type LNCaP cells, bcl-2, MDM2, p53, p21, p27, pRb, E2F1, and AR are downregulated and AKT, phosphoAkt and phosphoMAPK are upregulated. The molecular response of LNCaP-bcl-2 cells to AD was similar to wild-type LNCaP cells.

LNCaP-Res cells have the same growth rate in control medium (CM) or androgen deprived (AD) medium, whereas there is little growth of wild-type LNCaP cells in AD medium (**Figure 2**). LNCaP-BST (bcl-2 overexpressing) cells have an intermediate growth rate response to AD; LNCaP-MST (MDM2 overexpressing) cells respond similarly (not shown). LNCaP-BST cells were tested here because LNCaP-Res cells overexpress bcl-2 in AD medium (**Figure 3**) and bcl-2 overexpression is associated with reduced response to AD.

**Figure 2** also shows that androgen replacement using  $1 \times 10^{-10}$  M R1881 (AD+R; titrated to maximally reverse LNCaP growth inhibition from AD) reduced the growth rate suppression by AD in LNCaP and LNCaP-BST cells. However, the growth of LNCaP-Res cells in AD+R1881 medium was substantially reduced; androgen supplementation at this level was dramatically inhibited LNCaP-Res cell growth.

There were a number of changes in the molecular footprint of LNCaP-Res cells under the conditions of AD and AD+R1881 (**Figure 4**) that

Despite the lower levels of MDM2 after LNCaP-Res exposure to AD, these cells had higher levels of apoptosis by Caspase 3+7 assay to AS-MDM2+AD, compared to AS-MDM2 alone or AD alone (**Table 2**). These results have been replicated in Annexin V and clonogenic survival assays (data not shown), as well by measuring tumor growth in vivo using MRI to quantify tumor volume. Although bcl-2 is considerably elevated in LNCaP-Res cells grown in AD medium (**Figure 3**) and the apoptotic response pattern in **Table 2** is similar for LNCaP-Res and LNCaP-BST cells, there are fundamental differences in the growth and molecular responses to AD±R1881. Bcl-2 is but one of several factors dictating LNCaP-Res response to AD.

The apoptotic activity of AS-MDM2 under the condition of AD appears to be independent of bcl-2. Our results suggest that in response to AS-MDM2 + AD there is a slight increase in bax, the AR (androgen receptor) is substantially reduced and PUMA is relatively unaltered, as compared to AD alone (data not shown). The hypothesis is that the mechanism is related to effects on the AR, although alternative mechanisms are possible. The experiments for this paper are complete and the paper is in preparation.

*Task 2.* Define the molecular mechanisms underlying the changes in LNCaP cell killing in response to AD ± RT when MDM2 is suppressed or overexpressed.

- a. Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD and AS given simultaneously. Months 1-12.
- b. Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD given 2 d before AS. Months 13-24.
- c. Manipulation of gene expression to further enhance/replace the action of AD, RT, or AD+RT based on the Western results from the studies in years 1 and 2; for example, targeting p53 using adenoviral-p53, E2F-1 using adenoviral E2F-1, or bcl-2 using antisense bcl-2. Months 25-36

As described in prior annual reports, Most of the proposed LNCaP Western blot analyses were included in the two papers that were described above.<sup>1,5</sup> AS-MDM2 caused a reduction in MDM2, which was even further reduced by AD. P53 and p21 increased after AS-MDM2 or RT. Little effect was seen in bcl-2 and bax levels in response to AS-MDM2. We have also examined mRNA expression using the Oligo GE Array (SuperArray Bioscience Corp, Frederick, MD). A number of genes have been found to be increased greater than two fold with AS-MDM2 over the mismatch control. For example, bax was elevated to a greater degree than bcl-2. These experiments were not pursued further because of the interesting findings using Ad-E2F1 in wild type LNCaP cells and AS-MDM2 in LNCaP-Res cells (described in Task 1 above). As per Task 2c, we have published on the radiosensitization from adenoviral-E2F-1 (Ad-E2F-1) and described the protein expression changes that result.<sup>6</sup>

*Task 3.* Examine the degree and predictive value of MDM2 overexpression in diagnostic archival tissue specimens from patients treated with RT alone and RT + AD.

- a. MDM2 immunohistochemistry analysis of 110 cases from RTOG protocol 86-10. Months 1-6.
- b. Statistical analysis of MDM2 staining results from RTOG protocol 86-10. Months 7-10.
- c. MDM2 immunohistochemistry analysis of cases from RTOG protocol 92-02. Months 7-30.

- d. Statistical analysis of MDM2 staining results from RTOG protocol 92-02. Months 30-36.

The immunohistochemical staining and analysis of MDM2 expression in 109 diagnostic samples from patients treated in RTOG 86-10 (RT alone vs short term AD + RT) has been presented at a national meeting<sup>9</sup> and then published.<sup>10</sup> The nuclear staining of MDM2 expression was quantified manually and using an image analysis system (ACIS, ChromaVision, San Juan Capistrano, CA). The strongest relationship to outcome was found for the ACIS determination of the percentage of cells staining positive (PSP;  $p=0.06$  for distant metastasis in multivariate analysis), although a trend seen with the mean intensity score (MIS) as well.

In a larger study of 469 men treated in RTOG 92-02 (short term AD +RT vs long term AD + RT) MDM2 overexpression using the MIS was found to be a strong predictor of distant metastasis, independent of initial pretreatment PSA, Gleason score, T-stage, p53 and Ki-67. The results on MDM2 overexpression without the inclusion of p53 and Ki-67 were presented at the 2005 meeting of the American Radium Society (Barcelona, Spain).<sup>11</sup> The results with the inclusion of p53 and Ki-67 were presented at the 2005 meeting of ASTRO.<sup>12</sup> Our prior results have shown that both p53<sup>13</sup> and Ki-67<sup>12,14</sup> are strong predictors of distant metastasis in men treated with RT±AD, making the observations with MDM2 overexpression even more important. The paper is being prepared now.

## KEY RESEARCH ACCOMPLISHMENTS

- AS-MDM2 sensitizes androgen sensitive LNCaP prostate cancer cells to androgen deprivation, radiation and the combination.<sup>1,5</sup> These are the first reports to demonstrate such an interaction.
- Apoptosis appears to be the major cell death pathway affected by AS-MDM2.
- MDM2 overexpressing LNCaP-MST cells were more resistant to the sensitizing action of AS-MDM2, confirming the role of MDM2 in the development of prostate cancer cell resistance to androgen deprivation.
- AS-MDM2 enhances apoptosis of androgen insensitive (LNCaP-Res) cells in vitro and in vivo. This is the first study to demonstrate that AS-MDM2 continues to cause increased cell killing when combined with AD in prostate cancer cells no longer growth inhibited by AD. These data suggest that men with heterogeneous prostate cancers including cells with relative insensitivity to AD may still be eradicated by the combination of AS-MDM2 + AD.
- MDM2 is common in tumors from men with locally advanced prostate cancer and is associated with an increased risk of distant metastasis.<sup>10</sup> These are the first studies to demonstrate that MDM2 overexpression is an independent predictor of prostate cancer outcome, suggesting that prostate cancer patients who would benefit most from targeted MDM2 therapy may be selected.
- MDM2 overexpression is predictive of distant metastasis and mortality independent of conventional factors, treatment, p53 and Ki-67.<sup>12</sup> The findings are meaningful considering that p53 and Ki-67 are strong predictors of the outcome as well.<sup>13-15</sup>
- E2F1 overexpression using an adenoviral vector (Ad-E2F1) strongly sensitizes androgen sensitive and insensitive prostate cancer cells to RT.<sup>6</sup> Since MDM2 and E2F1 have opposing roles in a common apoptotic pathway, these data support the rationale for combining Ad-E2F1 and AS-MDM2 in future studies.



- The combination of AD + RT results in slower growth in men experiencing relapse biochemically.<sup>16,17</sup> These data support the use of the combination of AD + RT, and the strategy of combining this approach with molecular targeting agents.

### REPORTABLE OUTCOMES (Including Bibliography of Publications)

1. **Pollack A**, Mu Z, Hachem P, Agrawal S. Modulation of Prostate Cancer Cell Death in Response to Androgen Deprivation and Radiation by MDM2. International Conference on Translational Research, Lugano, Switzerland 3/17/03
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## CONCLUSIONS

The studies described indicate that MDM2 plays an important role in the response of prostate cancer cells to RT, AD and RT+AD. The in vitro experiments in LNCaP and LNCaP-MST cell lines illustrate the link between manipulating apoptosis and increasing overall cell killing by clonogenic assay. The in vivo investigations have been completed (paper in preparation), confirming that AS-MDM2 + AD and AS-MDM2 + AD + RT result in increased freedom from tumor volume and biochemical failure. These data confirm the in vitro results and are important step toward bringing this strategy into a clinical trial. Taken together, the in vitro and in vivo data indicate that AS-MDM2 holds promise as a therapeutic strategy for nearly every prostate cancer risk group. Those with localized favorable to intermediate risk disease may benefit from the use of lower doses of RT and consequently reduced side effects. Those with localized high risk disease are usually treated with AD+RT and have a significant risk of microscopic nodal and distant metastasis. The potentiation of the response of metastatic prostate cancer cells to AD by AS-MDM2 makes this approach particularly attractive. Our results in LNCaP-Res cells (paper in preparation) indicate that AS-MDM2 even has activity in cells that demonstrate no growth inhibition to AD and in bcl-2-overexpressing LNCaP cells that display less than wild-type LNCaP cell growth rate inhibition to AD. In addition, the demonstration that Ad-E2F1 sensitizes prostate cancer cells to RT supports the strategy of combining E2F1 overexpression and MDM2 suppression in future studies.

The analysis of MDM2 expression by immunohistochemistry in archival tissue from RTOG protocols 86-10 and 92-02 are concordant with the preclinical antisense studies. MDM2 overexpression is associated with an increased risk of distant metastasis and death, which is independent of whether the patients received RT alone, RT + short term AD or RT + long term AD. Moreover, the significance of MDM2 overexpression was also independent of p53 and Ki-67. MDM2 expression is turning out to be one of the most important determinants of outcome yet investigated. We now have a method not only for identifying men at high risk of treatment failure, but also for selecting men who would have the greatest potential benefit from therapeutically targeting MDM2.

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## APPENDIX

- Mu, Z., Hachem, P., Agrawal, S., Pollack, A. Antisense MDM2 Sensitizes Prostate Cancer Cells to Androgen Deprivation, Radiation, and the Combination. *Int. J. Radiation Oncology Phys.* **58**:336-343, 2004.
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## ANTISENSE MDM2 SENSITIZES PROSTATE CANCER CELLS TO ANDROGEN DEPRIVATION, RADIATION, AND THE COMBINATION

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**Purpose:** Antisense MDM2 (AS) sensitizes a variety of tumor cell types, including prostate cancer, to radiation and chemotherapy. We have previously described that AS enhances the apoptotic response to androgen deprivation (AD) and that this translates into a reduction in overall cell survival, as measured by clonogenic assay. Because AD + radiation (RT) is a key strategy for the treatment of men with high-risk prostate cancer, AS was tested for the ability to sensitize cells to the combination of AD+RT.

**Methods and Materials:** LNCaP cells were cultured *in vitro* in either complete, androgen deprived (AD), or AD+R1881 (synthetic androgen) medium for 2–3 days before AS was administered. Radiation at 5 Gy was given 18–24 h later. Processing of the cells after RT was done at 3 h for Western blots, 24 and 48 h for trypan blue dye exclusion, 18 h for Annexin V staining by flow cytometric analysis, 18 h for Caspase 3+7 quantification by fluorometric assay, and immediately for clonogenic survival measured 12–14 days later. There were 18 treatment groups that were studied: lipofectin control, AS, antisense mismatch (ASM), AD, AD+R1881, and RT in all possible combinations. Statistical comparisons between groups were accomplished with one-way analysis of variance using the Bonferroni test, considering all 18 groups.

**Results:** AS caused a reduction in MDM2 expression and an increase in p53 and p21 expression. Early cell death by trypan blue was found to be reflective of the apoptotic results by Annexin V and Caspase 3+7. AS caused a significant increase in apoptosis over the lipofectin control, AD, and RT controls. Apoptosis was further increased significantly by the addition of AD or RT to AS. When AS, AD, and RT were combined, there was a consistent increase in early cell death over AS+AD and AS+RT by all of the assay methods, although this increase was not significant. Overall cell death measured by clonogenic assay revealed synergistic cell killing of AS+RT beyond that of ASM+RT and RT alone, and AS+RT+AD beyond that of AS+RT, AS+RT+AD+R1881, ASM+RT+AD, and ASM+RT+AD+R1881.

**Conclusion:** AS sensitizes cells to AD, RT, and AD+RT and shows promise in the treatment of the full range of patients with prostate cancer. AS has the potential to sensitize the primary tumor to AD+RT and metastasis to AD. © 2004 Elsevier Inc.

Antisense, MDM2, Androgen deprivation, Radiation, Prostate cancer.

### INTRODUCTION

The combination of androgen deprivation (AD) plus radiation (RT) has become the standard for patients with high-risk prostate cancer. Despite the documentation of a survival improvement from this combination over RT alone in some series (1–3), there are still questions regarding the long-term efficacy over AD alone (4). An understanding of the molecular events that occur in the response of cells to AD and RT could lead to novel strategies that enhance cell killing in response to these agents, thereby allowing for the potential

to reduce toxicity through reduced exposure. It may be possible even to replace AD and RT altogether with less morbid alternative biologic therapies. Our approach has been to manipulate the apoptotic pathway.

Recently, we focused on MDM2 as a target for enhancing the apoptotic response of LNCaP cells to AD. The rationale was that MDM2 is overexpressed in 30–40% of prostate cancers (5, 6), MDM2 regulates p53 expression through a negative feedback loop (7), and p53 has been implicated in the apoptotic response of prostate epithelial cells to AD (8–12). An effective method for ablating MDM2 expression

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is through antisense MDM2 (AS) (13–16). Prior studies from our group have shown that AS+AD results in increased apoptosis over that seen by AS, AD, antisense mismatch (ASM), or ASM+AD (17). The pattern of increased early apoptotic cell death was mirrored in clonogenic survival assays, suggesting that overall cell death of LNCaP cells was significantly enhanced by the addition of AS to AD. Because AS has been shown to sensitize cells to RT and chemotherapy in a number of cell lines, it was hypothesized that AS will sensitize prostate cancer cells not only to AD and RT given individually, but also to AD+RT. Wild-type p53-expressing human LNCaP cells were chosen for the investigation of the effects of AS on AD+RT.

## METHODS AND MATERIALS

### *Antisense oligonucleotides*

The oligonucleotides were provided by Hybridon, Inc. (Cambridge, MA). The antisense MDM2 oligonucleotide (AS) and its mismatch control oligonucleotide (ASM) are 20-mer mixed-backbone oligonucleotides with following sequence (AS; 5'-UGACACCTGTTCTCACUCAC-3') and (ASM; 5'-UGTCACCCTTTTTCATUCAC-3'). They were stored as frozen aliquots at  $-20^{\circ}\text{C}$ .

### *Cell culture system*

LNCaP cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium-F12 medium, containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (complete medium [CM]), as described previously (18). Cells were typically cultured in complete medium before the culture conditions were altered. Androgen deprivation was achieved by culturing the cells in medium containing 10% charcoal-stripped serum (AD medium). Androgen was replaced by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at  $1 \times 10^{-10}$  M to AD medium (18).

### *Western blot analyses*

Protein levels of MDM2, p53, p21, Bcl-2, Bax, E2F1, pRb, and  $\beta$ -actin were analyzed after different treatments. Cells were cultured in complete, AD, or AD+R1881 medium for 3 days and incubated with 200 nM of AS or ASM in 4 mL culture medium for 24 h in the presence of 7  $\mu\text{g}/\text{mL}$  lipofectin (Invitrogen, Carlsbad, CA). Three hours after  $\gamma$ -irradiation to 5 Gy (RT) using a  $^{137}\text{Cs}$  irradiator (Model 81-14R, J.L. Shepherd & Associates, San Fernando, CA), cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS] with protease inhibitor cocktail set I [Calbiochem, San Diego, CA]) and were sonicated for 30 s on ice. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of protein were fractionated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and

5% nonfat milk) for 1 h at room temperature and were washed twice with the washing buffer (phosphate-buffered saline containing 0.1% Tween 20) for 5 min. The membranes were then incubated with the appropriate primary antibody: anti-MDM2 monoclonal antibody (mAb) at 1:1000; anti-p53 mAb at 1:1000; anti-p21 mAb at 1:1000; anti-Rb mAb at 1:1000, anti- $\beta$  actin at 1:5000 dilution or anti-E2F1 mAb at 1:1000 dilution (all antibodies from Calbiochem, San Diego, CA), anti-Bcl-2 mAb at 1:1000 (DAKO A/S, Carpinteria, CA), or anti-Bax polyclonal IgG at 1:1000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at  $4^{\circ}\text{C}$ . Membranes were washed and then incubated with 1:2000 diluted sheep anti-mouse IgG or donkey-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. After the washes were repeated, the proteins of interest were detected by the enhanced chemiluminescence reagents according to the manufacturer's directions (Amersham, Aylesbury, UK).

### *Trypan blue cell viability assay*

Early overall cell viability was assessed by trypan blue dye exclusion. Cells were seeded at  $5 \times 10^4$  cells/well in 24-well plates and cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then transfected with 200 nM of AS or ASM in the presence of lipofectin (7  $\mu\text{g}/\text{mL}$ ). After 24 h, cells were irradiated to 5 Gy. The percentage of dead cells was measured by trypan blue dye exclusion at 24 and 48 h after treatment; typical cumulative cell death rates after AS treatment were 37% and 52%. From these data, the 48-h time point was chosen to be representative.

### *Measurements of apoptosis*

Apoptosis was confirmed by Annexin V staining and Caspase 3+7 activity assays. LNCaP cells ( $2 \times 10^5$ ) were cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then incubated with 200 nM AS or ASM in the presence of lipofectin (7  $\mu\text{g}/\text{mL}$ ) for 18 h. Cells were then irradiated to 5 Gy. After 24 h, all cells (floating and attached) were harvested by trypsinization and labeled with Annexin V-PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies Inc., Burlingame, CA).

Caspase 3+7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured for 2–3 days in CM, AD medium, or AD+R1881 medium and then incubated with AS or ASM for 18 h. Different times for AS exposure and the delay in performing the assay after RT were tested, and 18-h times were found to be representative, without excessive activity. Cells were then irradiated to 5 Gy. After 18 h, a total of  $5 \times 10^4$  cells in 100  $\mu\text{L}$  culture medium were mixed with 100  $\mu\text{L}$  of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 h. Substrate cleavage was

Table 1. Western blot analyses of the effects of AD and/or RT on densitometry measurements of the expression of key proteins in the apoptotic pathway

Group	<i>n</i> *	CM+RT÷ CM	AD+RT÷ AD	AD+R1881+RT÷ AD+R1881
p53	5	6.8 ± 2.0	2.8 ± 0.6	4.5 ± 1.6
p21	3	3.0 ± 0.3	9.5 ± 2.5	7.7 ± 1.1
Bcl-2	5	0.9 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
Bax	3	0.5 ± 0.2	0.7 ± 0.1	1.1 ± 0.1
MDM2	4	11.8 ± 3.3	26.8 ± 7.5	13.9 ± 5.2
E2F1	3	1.1 ± 0.2	1.9 ± 0.3	1.9 ± 0.8
pRb	2	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.3

\* *n* = number of Western blot analyses done.

Note: The relative changes in band density measured by densitometry are shown as mean ± SEM.

quantified fluorometrically at 485-nm excitation and 538-nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems Inc., Franklin, MA). For a control, caspase 3+7 activity was inhibited by adding Ac-DEVD-CHO (Promega, Madison, WI) to the cell culture before the assay.

#### Radiation treatment and clonogenic assay

Cells were cultured in complete, AD, or AD+R1881 medium for 2–3 days and then incubated with 200 nM AS or ASM in the presence of lipofectin (7 µg/mL). After 24 h, cells were irradiated to 2, 4, and 6 Gy. Immediately after irradiation, cells were trypsinized and serially diluted, and known numbers of cells were replated into 100-mm dishes. The plates were incubated for 12–14 days and stained with 0.25% methylene blue. The colonies were counted using an automated counter (Imaging Products International, Inc., Chantilly, VA). The clonogenic survival results were corrected for differences in plating efficiency from the various culture conditions. The dilutions for clonogenic assay were done in triplicate, and the results were averaged together (intraexperimental averages). The data shown in the clonogenic survival table represent the average from multiple experiments (interexperimental average).

## RESULTS

#### Western blot analyses

MDM2 was identified as a potential target to enhance the response of prostate cancer cells to AD and RT through an investigation of the changes induced by these conditions in the expression of a variety of proteins involved in the apoptotic pathway. Table 1 displays the changes of MDM2, p53, p21, bcl-2, bax, E2F1, and pRb protein levels to AD ± RT, as determined by densitometry measurements of the resultant bands from Western blot analyses. The ratios of the band densities are shown. The average of 4 experiments of MDM2 revealed an 11.8-fold and a 26.8-fold increase in expression of MDM2 for CM+RT over CM and AD+RT over AD alone, respectively. When R1881 was added, the ratio of AD+RT over AD alone fell back to nearly the level

of the CM+RT over CM ratio. The changing level of MDM2 in response AD and RT was reflective of the changes in apoptosis under these conditions (18, 19). For these reasons, combined with the findings that p53 influences the apoptotic response of prostate epithelial cells to AD (8, 9), MDM2 was targeted using an antisense strategy.

Figure 1 displays representative Western blots showing that AS almost completely abrogated radiation-induced MDM2 expression in either complete, AD, or AD+R1881 medium, whereas ASM had little effect. The level of p53 increased after AS or RT treatment; ASM also increased the level of p53, as well as p21, but to a lesser degree. The mechanism for the slight increase in p53 levels after exposure to ASM is unclear, although in other Western blots, MDM2 seemed to be elevated from ASM treatment. The level of p21 was not increased by RT treatment, but was increased by AS treatment. AD alone had little effect on the protein levels of MDM2, p53, or p21. The expression of MDM2 seemed to be slightly higher for AS+AD+RT as compared to AS+AD, AS+RT, and AS+AD+R1881+RT. There was no obvious change in bcl-2 or bax expression by Western blot analysis in response to AS, AD, or RT (not shown).

#### Early cell death after AS ± AD ± RT treatment

The ability of AS to enhance the response of LNCaP cells to AD and/or RT was first evaluated using trypan blue dye exclusion. The cells were exposed to 200 nM AS, with or without AD, for 24 h, followed by γ-irradiation (5 Gy). A summary of three experiments measuring cell death 48 h after radiation is shown in Table 2. Eighteen treatment groups were analyzed together using analysis of variance. The statistics for the group comparisons are shown relative to the group above. Additional comparisons showed that AS resulted in significantly less cell death than AS+AD or AS+RT; these latter groups had about the same level of cell death. When R1881 was added to AS+AD, there was a reduction in cell death back to the level of AS. When AS was added to AD+RT, cell death was enhanced over all of the other groups, but the differences beyond that seen with AS+AD and AS+RT were not significant.

Direct measurements of apoptosis were performed to determine the contribution of apoptosis to early overall cell death that was quantified above by trypan blue staining. Apoptosis was measured directly by Annexin V binding. Cells were cultured in either complete, AD, or AD+R1881 medium for 48 h and then incubated with 200 nM AS or ASM for 18 h, followed by γ-irradiation (5 Gy). Twenty-four hours after irradiation, cells were prepared for Annexin V–PE and 7-AAD staining. Table 3 shows that early apoptosis (Annexin V–PE-positive and 7-AAD-negative) was higher from AS+AD (36.6% apoptosis) and AS+RT (32.7%) treatments over either AS (22.2%), AD (6.7%), or RT (3.9%) treatments given individually. These findings were significant (Table 2). However, there was no significant difference between AS+AD or AS+RT and AD+AS+RT, although the level of apoptosis was consistently higher in the AD+AS+RT group.

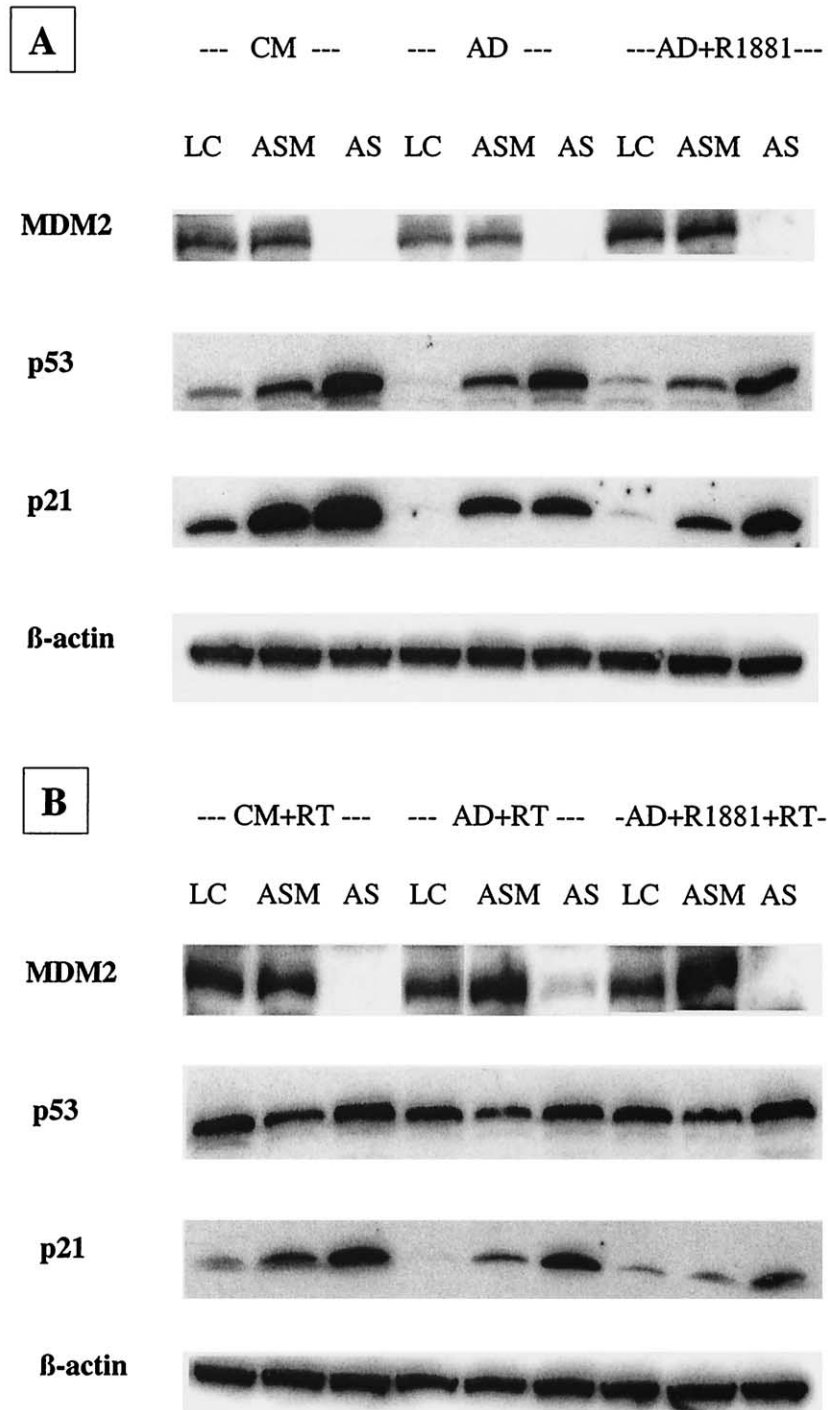


Fig. 1. Western blot analyses of LNCaP cells grown for 2–3 days in CM, AD, or AD+R1881 medium. AS or ASM was administered at 200 nM; 24 h later, RT at 5 Gy was given. The cells were harvested 3 h later, and the protein was extracted for analysis of MDM2, p53, p21, and  $\beta$ -actin levels. (A) Without RT; (B) With RT.

The pattern of apoptotic cell death observed by the Annexin V assay was very similar to that from the Caspase 3+7 assay. As shown in Table 4, Caspase 3+7 activity was increased from AS+AD or AS+RT as compared to AS, AD or RT treatments given singly. There was no significant increase in apoptosis from AS+AD+RT over that from AS+AD or AS+RT.

Caspase 3+7 activity was inhibited by the addition of R1881 to AS+AD to approximately the levels of AS alone. Moreover, the addition of specific caspase inhibitor Ac-DEVD-CHO (data not shown) reduced caspase 3+7 activity. These results suggest that AS accentuates LNCaP tumor cell apoptosis to AD and RT through p53 by activating caspase 3+7.



Table 2. Trypan blue quantification of early cell death

Treatment	Mean	SEM	p*
Lipofectin control	10.8	0.5	—
AS <sup>†</sup>	52.0	3.4	<0.0001
ASM	24.8	1.4	<0.0001
AD	21.3	1.4	1.000
AD+AS <sup>†,‡</sup>	71.0	3.9	<0.0001
AD+ASM	31.8	1.9	<0.0001
AD+R1881	15.5	2.1	0.226
AD+AS+R1881 <sup>†</sup>	57.0	5.6	<0.0001
AD+ASM+R1881	30.0	3.9	<0.0001
Lipofectin control+RT	21.8	2.2	1.000
AS+RT <sup>‡§</sup>	69.5	3.4	<0.0001
ASM+RT	30.8	3.7	<0.0001
AD+RT <sup>‡</sup>	26.3	2.2	1.000
AD+AS+RT <sup>§</sup>	82.3	3.5	<0.0001
AD+ASM+RT	39.3	4.2	<0.0001
AD+R1881+RT	19.3	1.1	0.02
AD+R1881+AS+RT <sup>§</sup>	69.0	4.1	<0.0001
AD+R1881+ASM+RT	35.8	6.7	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

\* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

<sup>†</sup> AD+AS vs. AS ( $p = 0.039$ ); AD+AS vs. AD+AS+R1881 ( $p = 0.855$ ).

<sup>‡</sup> AD+AS vs. AS+RT ( $p = 1.000$ ); AD+AS vs. AD+RT ( $p < 0.0001$ ).

<sup>§</sup> AD+AS vs. AD+AS+RT ( $p = 1.000$ ); AS+RT vs. AD+AS+RT ( $p = 1.000$ ); AD+AS+RT vs. AD+R1881+AS+RT ( $p = 1.000$ ).

#### Overall cell death by clonogenic cell survival assay

Clonogenic cell survival experiments were performed to determine whether the added, but not significant, early cell killing from apoptosis due to AS+AD+RT translates into a significant increase in overall cell killing, i.e., the cell killing manifested over time. The early cell death measurements by trypan blue and the apoptosis markers may not be representative of all cell death occurring over time. Figure 2 shows the clonogenic assay results for LNCaP cells grown for 2–3 days in CM and then treated with lipofectin alone, AS, or ASM for 24 h before RT. The cells were then replated immediately after RT at 2, 4, or 6 Gy. The results show LNCaP radiosensitization by AS at all RT dose levels, over the CM and ASM controls. Figure 3 reveals that radiosensitization was further enhanced when AD was added to AS and that this effect was reduced by R1881 supplementation. The radiosensitizing action of AS+AD was much greater than the minor effect observed from ASM+AD.

### DISCUSSION

Androgen deprivation and RT are central to the treatment of prostate cancer patients with high-risk prostate cancer. Even with the gains seen from this combination over single-modality therapy, the outcome of such high-

Table 3. Annexin V quantification of early apoptosis

Treatment	Mean	SEM	p*
Lipofectin control	3.4	0.7	—
AS <sup>†</sup>	22.2	0.5	<0.0001
ASM	7.4	0.4	<0.0001
AD	6.7	0.8	1.000
AD+AS <sup>†,‡</sup>	36.6	1.2	<0.0001
AD+ASM	12.1	1.1	<0.0001
AD+R1881	5.7	1.1	0.225
AD+AS+R1881 <sup>†</sup>	28.6	2.0	<0.0001
AD+ASM+R1881	10.7	1.4	<0.0001
Lipofectin control+RT	3.9	0.8	0.219
AS+RT <sup>‡§</sup>	32.7	1.4	<0.0001
ASM+RT	9.6	1.6	<0.0001
AD+RT <sup>‡</sup>	7.2	1.0	1.000
AD+AS+RT <sup>§</sup>	40.4	2.5	<0.0001
AD+ASM+RT	16.2	3.0	<0.0001
AD+R1881+RT	5.3	1.1	0.0001
AD+R1881+AS+RT <sup>§</sup>	34.3	1.8	<0.0001
AD+R1881+ASM+RT	12.2	2.1	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

\* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

<sup>†</sup> AD+AS vs. AS ( $p < 0.0001$ ). AD+AS vs. AD+AS+R1881 ( $p = 0.018$ ).

<sup>‡</sup> AD+AS vs. AS+RT ( $p = 1.000$ ); AD+AS vs. AD+RT ( $p < 0.0001$ ).

<sup>§</sup> AD+AS vs. AD+AS+RT ( $p = 0.106$ ); AS+RT vs. AD+AS+RT ( $p < 0.0001$ ); AD+AS+RT vs. AD+R1881+AS+RT ( $p = 0.964$ ).

risk patients remains rather poor. An understanding of the mechanisms of the interaction between AD and RT could lead to novel therapies that dramatically alter the failure profile.

Prior studies have indicated that p53 may have a role in the apoptotic response of prostate epithelial cells to AD (20). The results, however, have not been conclusive (21, 22). Little is known about why most prostate cancers respond to AD preferentially with a shift from cell proliferation to quiescence in the setting of minimal increases in apoptosis (23–28). There must be a key regulatory defect in the apoptotic pathway that preferentially shunts cells into quiescence instead of apoptosis. The data presented here point to MDM2. Of all of the proteins in the apoptotic pathway examined, MDM2 expression levels fluctuated in tandem with previously defined changes in apoptosis in response to AD+RT. We recently reported that in LNCaP cells grown *in vitro* (18) and in R3327-G Dunning rat prostate tumors grown *in vivo* (19), when AD precedes RT by 3 days, a supra-additive apoptotic response, over AD or RT given individually, is evidenced. Although supra-additive apoptosis was observed, the extent of the supra-additive response was rather minimal. The general lack of apoptosis seen in the response of prostate cancer cells to AD or RT alone, and

Table 4. Caspase 3+7 quantification of early apoptosis

Treatment	Mean	SEM	<i>p</i> *
Lipofectin control	114	16	—
AS <sup>†</sup>	335	19	<0.0001
ASM	199	25	0.169
AD	73	20	0.333
AD+AS <sup>†‡</sup>	504	7	<0.0001
AD+ASM	215	13	<0.0001
AD+R1881	109	29	1.000
AD+AS+R1881 <sup>†</sup>	349	20	<0.0001
AD+ASM+R1881	170	33	0.006
Lipofectin control+RT	89	10	1.000
AS+RT <sup>‡§</sup>	547	46	<0.0001
ASM+RT	259	27	<0.0001
AD+RT <sup>‡</sup>	112	15	0.071
AD+AS+RT <sup>§</sup>	610	35	<0.0001
AD+ASM+RT	302	15	<0.0001
AD+R1881+RT	90	21	<0.0001
AD+R1881+AS+RT <sup>§</sup>	491	50	<0.0001
AD+R1881+ASM+RT	218	38	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

\* Compared to group above, one-way ANOVA, Bonferroni test. The average of 3 experiments is shown.

<sup>†</sup> AD+AS vs. AS ( $p = 0.014$ ); AD+AS vs. AD+AS+R1881 ( $p = 0.039$ ).

<sup>‡</sup> AD+AS vs. AS+RT ( $p = 1.000$ ); AD+AS vs. AD+RT ( $p < 0.0001$ ).

<sup>§</sup> AD+AS vs. AD+AS+RT ( $p = 1.000$ ); AS+RT vs. AD+AS+RT ( $p = 1.000$ ); AD+AS+RT vs. AD+R1881+AS+RT ( $p = 0.571$ ).

the modest short-lived increase in apoptosis from the combination, suggest that apoptosis is being suppressed.

Under the conditions of AD+RT, the relative levels of MDM2 increase, as compared to AD alone or AD+RT+R1881. In light of the increase in apoptosis levels observed herein when MDM2 expression is suppressed, it seems that the increase in MDM2 in response to AD+RT is due to feedback regulation, such that MDM2 dampens what would otherwise be a very pronounced apoptotic response in normal prostate epithelial cells. Because overexpression of MDM2 is seen in 30%–40% of prostate cancers, the action of MDM2 on response to AD and/or RT has significant clinical implications.

Previously we found that the suppression of MDM2, through the use of antisense MDM2 oligonucleotides (AS), not only induces significant levels of apoptosis in LNCaP cells by itself, but also results in a pronounced enhancement in apoptosis when combined with AD (17). Those findings have been substantiated and extended in this communication. The main question posed here was whether AS sensitizes cells to the combination of AD+RT when all of the other possible treatments are considered. Antisense MDM2 has been shown to sensitize tumor cells to radiation (13). Radiosensitization in terms of the apoptotic response by AS was confirmed in LNCaP cells. Both AD+AS and RT+AS displayed greater levels of apoptosis than the sum of the individual treatments. When all three treatments were combined, there was a consistent, albeit insignificant, increase in apoptosis seen over AD+AS or RT+AS. Because apoptosis was measured at a single point in time and may not be reflective of overall cell killing, clonogenic cell survival assays were performed.

By clonogenic assay, AS has been shown previously to

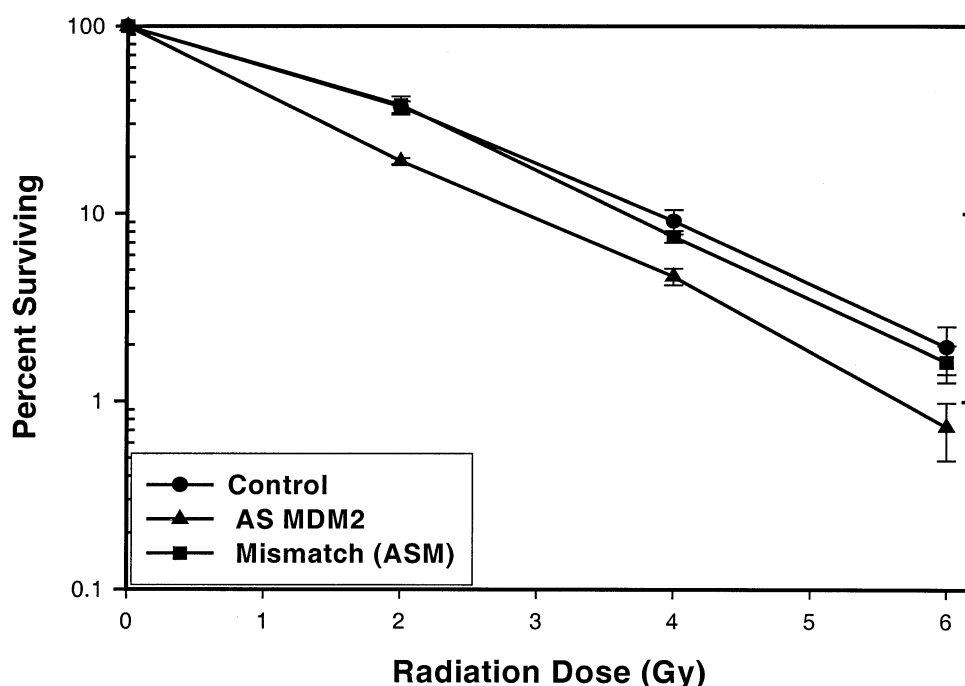


Fig. 2. Clonogenic assays of LNCaP cells cultured in CM alone or with AS or ASM (200 nM) added for 24 h before RT at 2, 4, or 6 Gy.

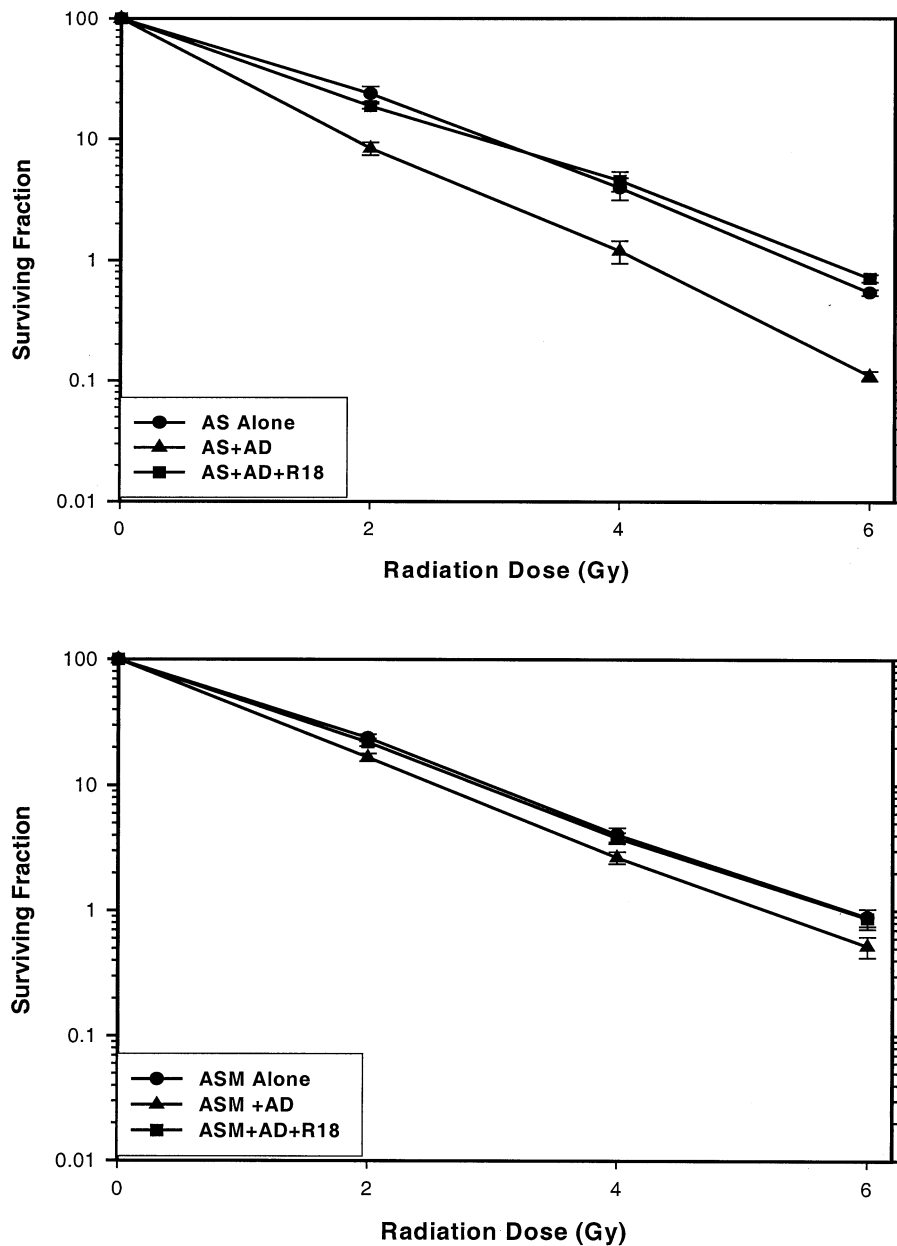


Fig. 3. Clonogenic assays of LNCaP cells cultured in CM or AD medium for 48–72 h and exposed to AS or ASM (200 nM) for 24 h before RT at 2, 4, or 6 Gy. AS = antisense MDM2; AD = androgen deprivation; R18 = synthetic androgen R1881; ASM = antisense mismatch.

significantly reduce clonogen survival when added to AD, as compared to each treatment applied individually. We show here that AS is also a potent radiosensitizer. Moreover, a further reduction of clonogen survival was evidenced when AS+AD+RT were given together, as compared to the controls (Fig. 3). The reduction in clonogenic cell survival was significant, and seemed to be greater than that observed by apoptosis alone. This could be related to the technical difficulty in summing apoptosis over time, which we did not attempt to do, or to other effects on cell survival, such as mitotic cell death. In either case, the data

substantiate the critical role of MDM2 in the response of prostate cancer cells to AD and RT.

## CONCLUSION

In summary, MDM2 is emerging as a central regulatory component in the cell death response of prostate cancer cells to AD and RT and has the potential to be manipulated therapeutically with AS. The hypothesized

mechanism for AS action is alteration of p53 expression via effects on MDM2 (the LNCaP cell line used is wild type for p53), although p53-independent effects may also contribute (7, 29). By enhancing the cell death response to AD, AS should improve cure rates by promoting cell

death in micrometastatic deposits, as well as reduce the number of clonogens at the primary site. The reduction in clonogens from AD+AS, when combined with the radiosensitizing effects of AS, makes this strategy ideal for the man with high-risk prostate cancer.

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# MDM2 as a Predictor of Prostate Carcinoma Outcome

## *An Analysis of Radiation Therapy Oncology Group Protocol 8610*

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**BACKGROUND.** The MDM2 oncoprotein promotes p53 degradation via ubiquitin, establishing negative feedback control of p53 and consequently affecting cell cycle arrest and apoptosis. The authors evaluated the association between MDM2 expression and local failure, distant metastasis (DM), cause-specific mortality, and overall mortality in men treated in Radiation Therapy Oncology Group 8610 with radiotherapy, with or without androgen deprivation.

**METHODS.** Of the 456 eligible and analyzable patients (parent cohort), adequate archival diagnostic tissue specimens from 108 patients were available for MDM2 analysis (MDM2 cohort). Cox proportional hazards multivariate analysis (MVA) was used to determine the relation of MDM2 to the endpoints. MDM2 overexpression was manually classified as > 5% nuclear staining. An image analysis system was also used to quantify the proportion of tumor nuclei with MDM2 staining (ACIS index) and staining intensity.

**RESULTS.** Overexpression of MDM2 by manual counts was seen in 44% ( $n = 47$ ) of the patients. In the manual count analysis, there was no significant relation between MDM2 overexpression and outcome. The ACIS index, using a cutoff point defined by the median value,  $\leq 3\%$  versus  $> 3\%$ , was related to 5-year DM rates in univariate analyses (32.6% vs. 45.8%;  $P = 0.057$ ) and MVA ( $P = 0.06$ ). The intensity of MDM2 staining was not significant.

**CONCLUSIONS.** MDM2 expression quantified by image analysis was weakly associated with DM. The cohort examined was relatively small and with larger patient numbers, MDM2 overexpression may emerge as a more significant covariate. *Cancer* 2005;104:962-7. © 2005 American Cancer Society.

**KEYWORDS:** MDM2, androgen deprivation, radiotherapy, distant metastasis.

The MDM2 oncoprotein, a ubiquitin ligase, binds to several apoptotic proteins including E2F-1, pRb, and p53, but is principally a negative regulator of p53. It is induced by p53, binds to its amino terminal transactivation domain, and consequently inhibits transcription of genes responsible for cell cycle arrest and apoptosis.<sup>1-3</sup> MDM2 oncogene overexpression has been seen in a variety of tumors,<sup>4</sup> including prostate carcinomas,<sup>5-7</sup> in which it has been observed in > 30% of men. It is associated with high-risk locoregional<sup>8</sup> and hormone-refractory disease.<sup>9</sup> In our experience, MDM2 suppression via antisense oligonucleotides sensitizes prostate tumor cells in vitro to radiotherapy (RT)<sup>10</sup> and androgen deprivation (AD).<sup>11</sup> Thus, MDM2 is a promising therapeutic target and the level of expression may be a useful marker of treatment outcome. To our knowledge, this is the first study to evaluate the predictive value of MDM2 overexpression in men with prostate carcinoma treated with RT.

Radiation Therapy Oncology Group (RTOG) protocol 8610 was a

Phase III randomized clinical trial designed to compare the effect of RT plus short-term neoadjuvant and concurrent (STAD) with RT alone.<sup>12</sup> The patients enrolled had locally advanced disease, with palpable tumors of surface area  $\geq 25$  cm<sup>2</sup>. Approximately one-third of the patients had Gleason score 8–10 disease and there was documented lymph node involvement in 8% of the patients. The purpose of the current analysis was to identify the relation of MDM2 expression to local failure (LF), distant metastasis (DM), cause-specific mortality (CSM), and overall mortality (OM).

## MATERIALS AND METHODS

### Patient Characteristics

RTOG 8610 has previously been described in detail.<sup>12</sup> The pretreatment diagnostic samples were sectioned and reviewed by the study pathologist (DJG). Of the 108 patient samples available for MDM2 analysis, the distribution of patients by Gleason score was 27 with a Gleason score of 2–6 and 80 with a Gleason score of 7–10 (1 patient was missing a Gleason score). The distribution of patients by clinical T classification was 29 with T2 and 79 with T3 disease. Sixty-two and 46 patients were assigned to RT alone and RT/STAD, respectively.

### Immunohistochemical Analysis

Sections best representing the tumor were cut 4- $\mu$ m thick onto poly-L-lysine slides from paraffin-embedded, formalin-fixed tissue specimens. The tissue specimen was then deparaffinized in xylene and rehydrated in a series of ethanol washes (95%) to a final distilled water step. Slides were then pressure cooked in an antigen retrieval citrate buffer solution (pH 6.0) for 50 minutes. After rinsing with water, the slides were covered with 3% hydrogen peroxide for 5 minutes at room temperature, then rinsed in Tris buffer, and humidified. The primary monoclonal MDM2 antibody (clone IF2, Zymed Laboratories, Inc., South San Francisco, CA; 1:100 dilution) was then overlaid. The slides were rinsed in Tris buffer, then incubated with Biotin (LSAB II kit; Dako Corporation, Carpinteria, CA) for 10 minutes, rinsed again as before, then incubated with Streptavidin for 10 minutes. After rinsing again with Tris buffer, chromagen (diaminobenzidine [DAB]; Research Genetics, Huntsville, AL) was applied for 5 minutes. The slides were then counterstained with commercially prepared hematoxylin (Dako Corporation) for 5 minutes, dehydrated, and coverslipped. All staining was performed on a Dako Autostainer. Positive controls with prostate carcinoma

tissue sections were used for comparison during tissue analysis.

Two investigators (L-YK and TA-S) reviewed the slides under a light microscope without knowledge of patient outcome. For the manual analysis,  $> 5\%$  dark brown nuclear tumor cell staining was considered positive, indicating overexpression of MDM2. This was considered a reasonable cutoff point to use because previous analyses considered any positive staining,<sup>8</sup>  $> 5\%$  staining<sup>13</sup> to  $\geq 20\%$  staining.<sup>7</sup>

The percentage of cells with nuclear staining (ACIS index) and the intensity of staining were also quantified using an image analysis system (ACIS, Clariant Inc., San Juan Capistrano, CA). A color threshold for brown (positive nuclei) and blue (negative nuclei) staining was set for every slide analyzed. When possible,  $\geq 3$  areas of interest in the tissue specimen visualized at  $\times 40$  magnification were designated for quantification. A final sample mean percent index (ACIS index) was derived by the computer software. Intensity of staining was scored on a gray scale of 0–255, in which 255 represented black.

The analysis of p53 by immunohistochemistry has been described previously in this patient population.<sup>14</sup> The staining methods used were similar. p53 was deemed positive when  $> 20\%$  of the tumor cells had nuclear staining, as quantified manually.

### Definition of Endpoints

The four endpoints examined were LF, DM, CSM, and OM. The details of these endpoints have been described previously.<sup>12,14,15</sup> Time to failure or death was measured from the date of randomization to the first reported date of failure.

### Statistical Analysis

There were 456 assessable patients in the parent cohort of RTOG 8610.<sup>12,16</sup> The MDM2 study cohort comprised 108 patients analyzed both manually and by ACIS. As of June 30, 2000, the median follow-up of all surviving patients in the study cohort was 9.3 years and the median follow-up of all entered patients was 6.7 years. The distributions of patient characteristics and treatment assignments were compared by the Pearson chi-square test and the Yates correction factor. Estimates of OM were derived using the Kaplan–Meier method,<sup>17</sup> whereas the cumulative incidence approach was used to estimate LF, DM, and CSM. Multivariate analysis (MVA) using Cox proportional hazard models was applied to each of the endpoints to identify the impact of MDM2.

There were 348 patients in the parent cohort in whom MDM2 was not quantified. Using the chi-square test, statistical comparisons were performed to

**TABLE 1**  
Distribution of all Patients by the Presence or Absence of MDM2 Data (*n* = 456)

Characteristics	Presence ( <i>n</i> = 108) (%)	Absence ( <i>n</i> = 348) (%)	<i>P</i> value <sup>a</sup>
GLSC			
2-6	27 (25)	102 (32)	0.21
7-10	80 (75)	220 (68)	
Unknown	1 (< 1)	26 (7)	
T-classification			
T2	29 (27)	108 (31)	0.41
T3	79 (73)	240 (69)	
Assigned treatment			
RT alone	62 (57)	168 (48)	0.10
RT + STAD	46 (43)	180 (52)	
p53			
Negative	70 (86)	36 (75)	0.10
Positive	11 (14)	12 (25)	
Unknown	27	300	

GLSC: Gleason score; RT: radiotherapy; STAD: short-term androgen deprivation.

<sup>a</sup> Chi-square statistics.

assess whether the distributions of patients by prognostic factors were different between the groups.

The MDM2 ACIS index and ACIS intensity score were modeled as continuous and categorical (using a cutoff point at the median value) variables in Cox proportional hazards models.

The interaction between MDM2 and p53 also led us to include the p53 data described in a previous study on RTOG 8610.<sup>14</sup> In that study, a cohort of 129 patients was analyzed for p53 positivity (overexpression) by immunohistochemistry. p53 overexpression was associated with an increase in the incidence of DM.

## RESULTS

We determined MDM2 overexpression in 108 (23.7%) of the 456 eligible patients in RTOG 8610. Table 1 shows the distribution of patients for whom MDM2 was (MDM2 cohort) and was not (other assessable patients in RTOG 8610) determined, according to pre-treatment characteristics and assigned treatment. There were no statistically significant differences in the distribution of patients by potential prognostic factors between these two groups. Table 2 displays the distribution of patients in the MDM2 cohort by MDM2 manual count results (5% cutoff point) and patient characteristics. The only significant finding was that MDM2 overexpression was significantly associated with higher Gleason scores. Forty (85%) patients with MDM2 overexpression had a Gleason score of 7-10, whereas 7 (15%) patients had a Gleason score of 2-6 (*P*

**TABLE 2**  
Distribution of Patients by MDM2 Manual Count Results

Characteristics	Negative ( <i>n</i> = 61) (%) <sup>a</sup>	Positive ( <i>n</i> = 47) (%)	<i>P</i> value <sup>b</sup>
Age (yrs)			
<75	46 (75)	35 (74)	0.91
≥75	15 (25)	12 (26)	
GLSC			
2-6	20 (33)	7 (15)	0.029
7-10	40 (67)	40 (85)	
T-classification			
T2	17 (28)	12 (26)	0.79
T3	44 (72)	35 (74)	
Assigned treatment			
RT alone	33 (54)	29 (62)	0.43
RT + STAD	28 (46)	18 (38)	
p53			
Negative	34 (83)	36 (90)	0.35
Positive	7 (17)	4 (10)	
Unknown	20	7	

GLSC: Gleason score; RT: radiotherapy; STAD: short-term androgen deprivation.

<sup>a</sup> One patient in the negative MDM2 group was missing the Gleason score.

<sup>b</sup> Chi-square statistics.

**TABLE 3**  
Univariate Analysis Results for the MDM2 Cohort (*n* = 108)<sup>a</sup>

Local failure RR (95% CI)	Distant metastasis RR (95% CI)	Cause-specific mortality RR (95% CI)	Overall mortality RR (95% CI)
0.92 (0.49-1.76) <i>P</i> = 0.81	1.49 (0.87-2.56) <i>P</i> = 0.15	1.32 (0.70-2.49) <i>P</i> = 0.40	1.12 (0.71-1.74) <i>P</i> = 0.63

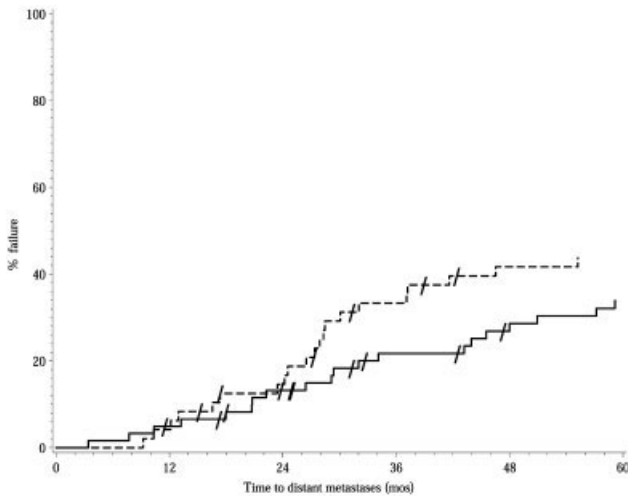
RR: relative risk; 95% CI: 95% confidence interval.

<sup>a</sup> *P* values were derived from the chi-square test.

= 0.029). MDM2 overexpression was not associated with age, clinical stage, assigned treatment, or p53 status.

The univariate analysis results for the MDM2 cohort are shown in Table 3. Although there was no significant relation between the MDM2 manual count results and outcome, MDM2 overexpression was associated with a 5-year DM rate in univariate analysis of 42.6% versus 28.6% when MDM2 was not overexpressed (*P* = 0.15) (Fig. 1). This observation may be clinically meaningful, given the relatively small sample of patients. The analyses, with respect to DM and the other endpoints tested, may not have been adequately powered to detect a difference in MDM2 expression. For the end point of DM, the power to detect the risk observed in the univariate analysis (relative risk [RR] = 1.49) was 31%. In the MVA (Table 4), controlling for Gleason score, p53 status, and assigned treatment, the





**FIGURE 1.** Survival curve of distant metastasis by MDM2 manual count results, using cumulative incidence estimates. Solid line: negative MDM2 manual counts; dashed line: positive MDM2 manual counts.

**TABLE 4**  
Multivariate Analysis of Distant Metastasis; MDM2 Manual Count results

Variable <sup>a</sup>	Group	RR (95% CI) <sup>b</sup>	P value <sup>c</sup>
MDM2	Positive	1.60 (0.82–3.10)	0.17
GLSC	7–10	2.66 (1.07–6.63)	0.0353
STAD	Yes	0.89 (0.47–1.68)	0.71
p53	Positive	2.67 (1.17–6.10)	0.0199

RR: relative risk; 95% CI: 95% confidence interval; GLSC: Gleason score; STAD: short-term androgen deprivation.

<sup>a</sup> All variables were dichotomous.

<sup>b</sup> A relative risk ratio of 1 indicates no difference between the two subgroups.

<sup>c</sup> P value was derived from the chi-square test using the Cox proportional hazards model.

association of the MDM2 manual count results with DM was slightly weaker ( $P = 0.17$ ).

The MDM2 manual count results were obtained using a  $> 5\%$  cutoff point for overexpression. A range of cutoff points have been used in the past.<sup>7,8,13</sup> The rationale for using this particular cutoff point was that it is clearly above background and has been used before.<sup>13</sup> However, the results were not statistically significant and there is the possibility that it is not the optimal cutoff point. Hence, we proceeded to use an image analysis system to more precisely quantify the proportion of tumor cells with nuclear MDM2 staining (ACIS index). A median ACIS index of 3.0% (range, 0–26.0%) was obtained. Table 5 shows the relation of the ACIS index to the manual results. When we compared the 75% quartile cutoff point of 5% with the equivalent manual results, there were some discrepancies: 3 patients, scored negative in manual analysis

**TABLE 5**  
Distribution of Manual Vs. ACIS Index Results

ACIS index	Manual		Total	P value <sup>a</sup>
	Negative (n = 61)	Positive (n = 47)		
≤ 1.0	28 (46)	0	28	< 0.0001
> 1.0	33 (54)	47 (100)	80	
≤ 3.0	54 (89)	5 (11)	59	< 0.0001
> 3.0	7 (11)	42 (89)	49	
≤ 5.0	58 (95)	24 (51)	82	< 0.0001
> 5.0	3 (5)	23 (49)	26	

<sup>a</sup> Chi-square statistics.

due to extremes in staining intensity, were scored  $> 5\%$  by ACIS. This is likely because of the ability of ACIS to more accurately score a wide range of staining intensities. Also, 15 of the 24 patients scored positive manually, were scored 5% exactly by ACIS.

The three cutoff points were then applied in univariate analysis to the four endpoints. A relation was seen between the median 3% ACIS index cutoff point and DM (Table 6). MDM2 overexpression in  $\leq 3\%$  of tumor cells was associated with a 5-year DM rate of 32.6% versus 45.8% when  $> 3\%$  had overexpression ( $P = 0.057$ ) (Fig. 2). A similar level of significance was seen in the MVA (RR = 1.85,  $P = 0.06$ ) (Table 7). p53 positivity and a Gleason score of 7–10 were significantly associated with DM (RR = 2.68,  $P = 0.02$ ; RR = 2.7,  $P = 0.03$ ). When the MDM2 ACIS index was used as a continuous variable in MVA, no relation to DM or the other endpoints was observed in MVA.

Finally, MVAs for the MDM2 ACIS intensity score, modeled both as a continuous variable and by the median cutoff point (162 relative units), suggested a relation with DM when used as a continuous variable (continuous:  $P = 0.10$ ; cutoff point:  $P = 0.97$ ). The ACIS intensity score was not associated with any other end point.

## DISCUSSION

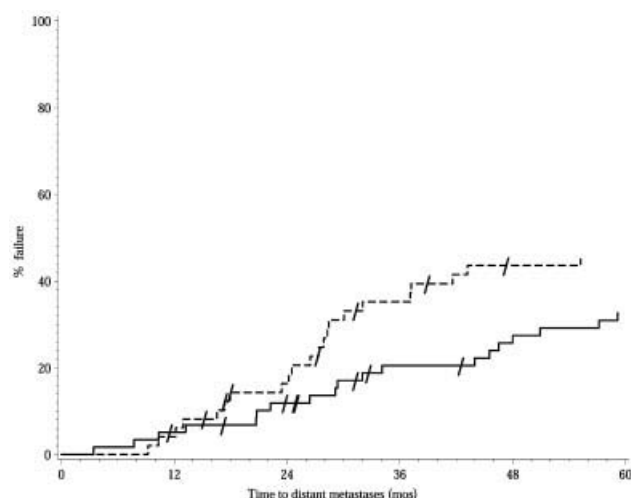
MDM2 is a key regulator of apoptosis through its interactions with p53, E2F1, pRB, and other proteins.<sup>18,19</sup> We described recently that the apoptotic response of prostate carcinoma cells to AD and/or RT was significantly affected by the level of MDM2 expression<sup>10,11</sup> in prostate carcinoma cell lines. Antisense MDM2 is available as a potential therapeutic adjunct to AD and RT. We investigated the expression of MDM2 in men treated with RT, with and without STAD, to determine whether MDM2 overexpression is predictive of patient outcome, as a prelude to targeting men with antisense MDM2 in future trials.



**TABLE 6**  
Univariate Analysis of the MDM2 ACIS Index<sup>a</sup>

End point	MDM2 ACIS cutoff point	No. of patients	Failures	RR <sup>b</sup> (95% CI)	P value <sup>c</sup>	5-yr rate (%)	5-yr (95% CI)
LF	≤ 3.0	59	21	1.07 (0.57–2.03)	0.83	25.4	(14.2–36.7)
LF	> 3.0	49	18			23.0	(10.9–35.7)
DM	≤ 3.0	59	26	1.69 (0.98–2.91)	0.057	32.6	(20.4–44.8)
DM	> 3.0	49	28			45.8	(31.4–60.1)
CSM	≤ 3.0	59	19	1.29 (0.68–2.44)	0.43	18.6	(8.6–28.7)
CSM	> 3.0	49	19			27.1	(14.4–39.9)
OM	≤ 3.0	59	42	1.13 (0.72–1.77)	0.59	33.9	(21.6–46.2)
OM	> 3.0	49	36			41.6	(27.4–55.8)

RR: relative risk; 95% CI: 95% confidence interval; LF: local failure; DM: distant metastasis; CSM: cause-specific mortality; OM: overall mortality.

<sup>a</sup> The MDM2 ACIS index indicator was coded as 0, cutoff point or lower; 1, higher than the cutoff point.<sup>b</sup> A relative risk ratio of 1 indicates no difference between the two subgroups.<sup>c</sup> P value was derived from the chi-square test using the Cox proportional hazards model.**FIGURE 2.** Survival curve of distant metastasis by MDM2 ACIS index at the 3% cutoff point, using cumulative incidence estimates. Solid line: ACIS index ≤ 3.0; dashed line: ACIS index > 3.0.

To our knowledge, little is known regarding the abnormal expression of MDM2 in prostate carcinoma, as it relates to other prognostic variables and patient outcome. Osman et al.<sup>7</sup> found MDM2 overexpression in 33% of 86 patients who received radical prostatectomy. MDM2 expression was not related to p53 expression, but was associated with advanced stage. No relation was observed between MDM2 expression and biochemical failure. Leite et al.<sup>8</sup> found that MDM2 was overexpressed in > 40% of 118 men who underwent radical prostatectomy and such overexpression was associated strongly with increased tumor volume ( $P = 0.001$ ) and weakly with a higher proliferation index ( $P = 0.046$ ) and higher tumor stage ( $P = 0.054$ ). MDM2 was not associated with p53. In our study, which is the first to investigate such relations in men treated with

**TABLE 7**  
Multivariate Analysis of Distant Metastasis; with the MDM2 ACIS Index Results

Variable <sup>a</sup>	Group	RR (95% CI) <sup>b</sup>	P value <sup>c</sup>
MDM2	> 3.0	1.85 (0.97–3.56)	0.06
GLSC	7–10	2.70 (1.09–6.71)	0.0328
STAD	Yes	0.89 (0.47–1.68)	0.72
p53	Positive	2.68 (1.18–6.07)	0.0181

RR: relative risk; 95% CI: 95% confidence interval; GLSC: Gleason score; STAD: short-term androgen deprivation;

<sup>a</sup> All variables were dichotomous.<sup>b</sup> A relative risk ratio of 1 indicates no difference between the two subgroups.<sup>c</sup> P value was derived from the chi-square test using the Cox proportional hazards model.

RT with or without STAD, nuclear MDM2 overexpression was observed in 44% of patients. MDM2 overexpression also was not related to p53 expression, but was related to a higher Gleason score and weakly to DM. The lack of a relation between p53 and MDM2 expression is possibly caused by a lack of feedback from mutant p53.

Our analysis of MDM2 expression was performed in 2 phases. First, we performed manual counts, assigning an incidence of > 5% tumor nuclear positivity to represent overexpression. Of the 3 previous reports of men with prostate carcinoma, the categorization of positive overexpression ranged from any nuclear staining to > 5% to ≥ 20%.<sup>7,8,13</sup> A value of > 5% seemed reasonable, as this could be easily recognized as being above background. However, the cutoff point of 5% is somewhat arbitrary.

The determination of the relation of the percentage tumor cells demonstrating MDM2 staining was quantified more precisely using an image analysis system. The resulting ACIS index, while correlating with

the manual count results, was more strongly related to the outcome measure of DM. The median ACIS index was 3% and this was chosen as the cutoff point. The ACIS index at the median cutoff point was related to DM in both univariate and multivariate analyses and MVA, although statistical significance at the  $P < 0.05$  level was not obtained ( $P = 0.06$ ). Likewise, the ACIS staining intensity was also related to DM, albeit more weakly. The results are promising in that such associations were seen even with relatively few patients and p53 included in the analysis.

The relation between MDM2 overexpression and DM in men treated with RT with or without AD may be clinically meaningful and should be further investigated in a larger cohort. The predictive value of MDM2 should also be investigated in a more contemporary group of men treated in the prostate-specific antigen era.

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## BIOLOGY CONTRIBUTION

 ADENOVIRAL-E2F-1 RADIOSENSITIZES p53<sup>wild-type</sup> AND p53<sup>null</sup> HUMAN PROSTATE CANCER CELLS

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**Purpose:** E2F-1 is a transcription factor that enhances the radiosensitivity of various cell lines by inducing apoptosis. However, there are conflicting data concerning whether this enhancement is mediated via p53 dependent pathways. Additionally, the role of E2F-1 in the response of human prostate cancer to radiation has not been well characterized. In this study, we investigated the effect of Adenoviral-E2F-1 (Ad-E2F-1) on the radiosensitivity of p53<sup>wild-type</sup> (LNCaP) and p53<sup>null</sup> (PC3) prostate cancer cell lines.

**Methods and Materials:** LNCaP and PC3 cells were transduced with Ad-E2F-1, Adenoviral-Luciferase (Ad-Luc) control vector, or Adenoviral-p53 (Ad-p53). Expression of E2F-1 and p53 was examined by Western blot analysis. Annexin V and caspase 3 + 7 assays were performed to estimate the levels of apoptosis. Clonogenic survival assays were used to determine overall cell death. Statistical significance was determined by analysis of variance, using the Bonferroni method to correct for multiple comparisons.

**Results:** Western blot analysis confirmed the efficacy of transductions with Ad-E2F-1 and Ad-p53. Ad-E2F-1 transduction significantly enhanced apoptosis and decreased clonogenic survival in both cell lines. These effects were compounded by the addition of RT. Although E2F-1-mediated radiosensitization was independent of p53 status, this effect was more pronounced in p53<sup>wild-type</sup> LNCaP cells. When PC3 cells were treated with Ad-p53 in combination with RT and Ad-E2F-1, there was at least an additive reduction in clonogenic survival.

**Conclusions:** Our results suggest that Ad-E2F-1 significantly enhances the response of p53<sup>wild-type</sup> and p53<sup>null</sup> prostate cancer cells to radiation therapy, although radiosensitization is more pronounced in the presence of p53. Ad-E2F-1 may be a useful adjunct to radiation therapy in the treatment of prostate cancer. © 2005 Elsevier Inc.

Adenoviral gene therapy, E2F-1, Prostate cancer, Radiation, Apoptosis.

## INTRODUCTION

E2F-1 is a transcription factor with multiple functions. Depending on the cellular milieu and predominant signal, it can act as either an oncogene or tumor suppressor (1, 2). Overexpression of E2F-1 in the presence of Ras mutations has led to malignant transformation (3). Singh and colleagues demonstrated the ability of E2F-1 to transform rat embryo fibroblasts (4). However, other studies have suggested an opposing role of E2F-1. In animal models, Field *et al.* (5) and Yamasaki *et al.* (6) observed that E2F-1 knockout mice have an increased propensity to form tumors. Through interactions with various cell cycle regulators, it can act as a tumor suppressor by mediating cell cycle arrest, DNA repair, or apoptosis (7, 8).

Gene transfection experiments have demonstrated the ability of E2F-1 overexpression to induce tumor regression (9). Additionally, E2F-1 overexpression has been shown to enhance cellular radiosensitivity and increase cell death via apoptosis in certain cell lines (10–12). Even in cells with intact native E2F-1, exogenous overexpression of E2F-1 can also lead to cell-cycle arrest or apoptosis (13–15). Although it is clear that E2F-1 plays a central role in cell-cycle regulation and DNA repair, its function in prostate cancer is less certain (16). Moreover, the potential of E2F-1 administered via a gene therapy vector in conjunction with radiation has never been examined.

P53 is a much-studied tumor suppressor gene with some mechanisms of action analogous to E2F-1. It has been described as “guardian of the genome,” regulating cell-

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cycle progression, promoting repair of sublethal DNA damage, and inducing cell death when alterations are irreparable (17–19). Tumors with p53 mutations have been observed to be more aggressive and resistant to many therapeutic modalities, including radiation (20–25). As with E2F-1 gene transfer strategies, introduction of p53 into p53<sup>wild-type</sup>, p53<sup>null</sup>, or p53 mutant cell lines also enhances radiation response (26–32).

In this study, we investigated the effects of Adenoviral-E2F-1 (Ad-E2F-1) and Ad-p53 gene therapy on the responses of prostate cancer cells to radiation. Specifically, we asked the question: Does Ad-E2F-1 sensitize prostate cancer cells to radiation, and, if so, to what extent is this effect dependent on p53? The effect of Ad-E2F-1 on cell killing from radiation was examined in the p53<sup>wild-type</sup> LNCaP and p53<sup>null</sup> PC3 human prostate cancer cell lines. Transduction experiments with both Ad-p53 and Ad-E2F-1 were performed to determine the effect of p53 replacement on the radiation response of PC3 cells to E2F-1 gene therapy.

## METHODS AND MATERIALS

### Cell culture

LNCaP and PC-3 cells from American Type Culture Collection (Rockville, MD) were maintained in Dulbecco's modified Eagle F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 4 mM glutamine. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### Transduction and protein expression analyses

Approximately  $5 \times 10^5$  cells were plated on 10-cm dishes in duplicate for approximately 48 h. Adenovirus-5 (CMV promoter) constructs incorporating the E2F-1 (Ad-E2F-1) (33), p53 (Ad-p53) (31), and Luciferase (Ad-Luc) (32) genes were used to transduce cells at a multiplicity of infection (MOI) of 10, 25, or 50. Twenty-four hours after gene transduction, one set was irradiated with 6 Gy and reincubated for approximately 3 h while the duplicate set received no radiation therapy (RT). Cells were then harvested and lysed using buffer (50 mM Tris pH 7, 2% sodium dodecyl sulfate) containing proteinase inhibitors.

Western blot analyses were performed to confirm the success of transduction. Approximately 50–70 µg of protein from each cell lysate was electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel. After transfer onto a polyvinylidene difluoride membrane in a transblot apparatus and blocking with 5% low-fat dried milk, the blots were incubated overnight at 4°C with mouse monoclonal antibodies to E2F-1 (Oncogene, La Jolla, CA), pRb (Oncogene), p53 (Santa Cruz Biotech, Santa Cruz, CA), or p21 (Oncogene) at 0.1% antibody concentration in milk-blocking buffer. The membranes were washed and labeled with an anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham, Buckinghamshire, UK) at room temperature for approximately 1 h. Detection by chemiluminescence was performed following the standard protocol in ECL user's guide (Amersham).

### Measurement of apoptosis

Annexin V and Caspase-3 + 7 assays were performed to determine whether E2F-1 mediates cell killing via apoptosis. For each

assay,  $5 \times 10^5$  cells were transduced with Ad-E2F-1. Eighteen hours after gene transduction, one set was irradiated and reincubated while the other received no irradiation. After an additional 48 h, cells were harvested for Annexin V or caspase 3 + 7 assay. For the Annexin V assay (Guava Technologies Inc., Burlingame, CA), cells were labeled with Annexin V-Phycoerythrin (Annexin V-PE) and 7-amino-actinomycin D (7AAD) according to the manufacturer's instructions, and analyzed by flow cytometry on a GuavaPC personal flow cytometer. The cells that stained for Annexin V-PE and did not stain with 7AAD were considered to be in early apoptosis and the percentages of these cells are displayed in the tables. Caspase-3 + 7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE Homogeneous Caspase-3 + 7 Assay kit; Promega, Madison, WI). Harvested cells were mixed with 100 µL of Homogenous Caspase-3 + 7 reagent in 96-well plates and incubated at room temperature for 18 h. Substrate cleavage was quantified fluorometrically at 485-nm excitation and 538-nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystem Inc., Franklin, MA).

### Clonogenic survival

The techniques for clonogenic survival assays have been described previously (34). For clonogenic survival assays, four sets of approximately  $5 \times 10^5$  cells were plated onto sterile 10-cm dishes. Typically, after 48 h,  $2 \times 10^6$  cells in each dish were available for gene transduction. The Ad-E2F-1, Ad-p53, and Ad-Luc vectors were maintained and diluted in phosphate-buffered saline until transduction. The cells in each dish were washed in phosphate-buffered saline to remove any residual serum that might bind viral particles and impede transduction. Appropriate dilutions of Ad-E2F-1 or Ad-p53 vector in 1 mL of solution were gently placed onto the monolayer of cells in each dish and incubated for 1 h. Control dishes with medium alone or with Ad-Luc were exposed to identical conditions. After incubation, 4 mL of control medium with serum was added to each dish and incubated overnight. At 24 h after viral exposure, three sets of dishes at each RT dose level were irradiated with a high dose rate cesium unit (<sup>137</sup>Cs irradiator, Model 81-14R, JL, Shepherd & Associates, San Fernando, CA) to a total of 2, 4, or 6 Gy. Immediately after irradiation, cells were trypsinized, serially diluted, replated into 100-mm dishes, and incubated. After 14 days, colonies were stained with methylene blue and counted. Cell survival was adjusted for plating

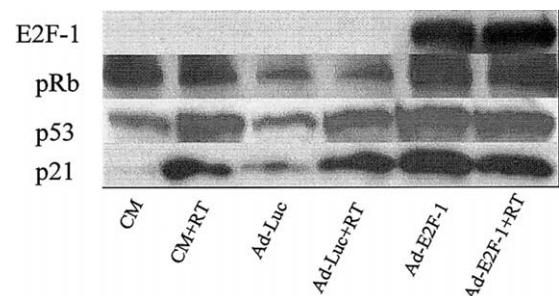


Fig. 1. LNCaP Western blots. LNCaP cells were transduced with 25 multiplicity of infection of Ad-E2F-1 or Ad-Luc. After 24 h of gene transduction, cells were irradiated with 6 Gy and lysed 3 h later. Abbreviations: CM = control medium; RT = radiation therapy; Ad-E2F-1 = Adenoviral-E2F-1; Ad-Luc = Adenoviral Luciferase.



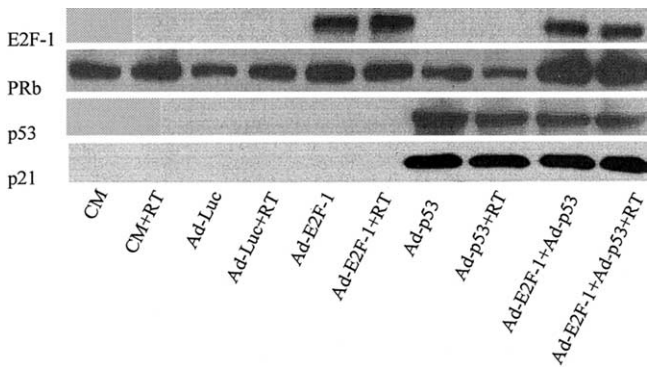


Fig. 2. PC3 Western blots. PC3 cells were transduced with 50 multiplicity of infection of Ad-E2F-1, Ad-p53, or both. Ad-Luc and control medium alone served as controls. After 24 h of gene transduction, cells were irradiated with 6 Gy and lysed 3 hours later. Abbreviations: CM = control medium; RT = radiation therapy; Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1; Ad-p53 = Adenoviral-p53.

efficiency. For each radiation dose and viral titer, five experiments were performed, and the results were averaged.

Statistical analysis

Statistical significance between groups was assessed using analysis of variance, correcting for multiple comparisons using the Bonferroni method. Differences were considered statistically significant at the  $p < 0.05$  level.

RESULTS

The success of E2F-1 transduction by Ad-E2F-1 in LNCaP cells was validated by Western blots. Figure 1 shows the expression of E2F-1, pRb, p53, and p21 in LNCaP cells treated with Ad-E2F-1 (25 MOI) or Ad-Luc (25 MOI), with or without 6 Gy single-dose RT. E2F-1 expression was evident in LNCaP cells transduced with Ad-E2F-1. The addition of RT did not appear to significantly increase

E2F-1 expression. Although E2F-1 expression was not evident in cells treated with Ad-Luc or nonvector containing control medium (CM), this is the result of the chemiluminescence exposure conditions used in this study. Other experiments using the same cell line, but with different chemiluminescence exposure conditions, showed E2F-1 expression when these cells were incubated in CM or with Ad-Luc (data not shown). Overexpression of E2F-1 resulted in slight increases in p53 and p21 expression over that of the CM and the Ad-Luc controls. This effect of Ad-E2F-1 was not seen on pRb expression, although pRb expression was reduced by Ad-Luc exposure. The addition of RT enhanced levels of p53 and p21 in cells incubated in CM or with Ad-Luc. In the presence of E2F-1 overexpression, p53 and p21 expression were not further enhanced by RT.

Western blots of E2F-1, pRb, p53, and p21 expression in PC3 cells are shown in Fig. 2. PC3 cells were transduced with Ad-E2F-1 (50 MOI), Ad-p53 (50 MOI), or both (50 MOI each), with or without 6 Gy RT. Incubation in CM or transduction with Ad-Luc (50 MOI) served as controls. In PC3 cells, E2F-1 expression was detectable in Ad-E2F-1 transduced cells but not in the others under the chemiluminescence exposure conditions used here. Other experiments using the same cell line, but with different chemiluminescence exposure conditions, showed E2F-1 expression when these cells were incubated in CM or with Ad-Luc (data not shown). Neither Ad-p53 nor RT significantly altered E2F-1 levels. pRb protein level was not affected by RT, but was slightly reduced by Ad-Luc, slightly increased by Ad-E2F-1, reduced by Ad-p53, and most obviously increased by Ad-E2F-1 plus Ad-p53. pRb expression was not affected by RT. As expected, p53 was not expressed in the p53<sup>null</sup> PC3 cell line. Transduction with Ad-p53 resulted in p53 expression that was independent of RT or Ad-E2F-1 exposure. The expression of p21 paralleled that of p53.

Levels of apoptosis in LNCaP and PC3 cells were determined using Annexin V and caspase 3 + 7 assays (Table 1).

Table 1. Apoptosis assays in LNCaP and PC3 prostate cancer cells

Groups	LNCaP				PC3			
	Annexin V*	Bonferroni test†	Caspase 3 + 7*	Bonferroni test†	Annexin V*	Bonferroni test†	Caspase 3 + 7*	Bonferroni test†
Control	5.4 ± 0.8	6	204 ± 68	1, 6	5.2 ± 1.9	6	65 ± 25	6
Ad-Luc	6.8 ± 2.9	7	446 ± 225	2, 7	5.7 ± 2.2	7	128 ± 57	7
Ad-E2F-1	10.4 ± 2.5	5	1507 ± 472	1, 2, 3, 4	4.1 ± 1.5	3, 5	723 ± 408	5
Control + RT	8.0 ± 1.4	8	440 ± 253	3, 8	8.2 ± 2.6	3	137 ± 70	8
Ad-Luc + RT	9.9 ± 1.2	9	499 ± 317	4, 9	8.1 ± 1.6	NS	194 ± 119	9
Ad-E2F-1 + RT	18.1 ± 3.1	5, 6, 7, 8, 9	2128 ± 77	6, 7, 8, 9	10.9 ± 1.8	5, 6, 7	1516 ± 782	5, 6, 7, 8, 9

Abbreviations: RT = radiotherapy; Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1.  
\* Annexin V (percent of Annexin V-PE positive and 7AAD negative) and Caspase 3 + 7 (relative fluorescence units) apoptosis assay values are tabulated as mean of five experiments ± standard deviation.  
† Bonferroni test: 1,  $p < 0.05$  for Ad-E2F-1 vs. control; 2,  $p < 0.05$  for Ad-E2F-1 vs. Ad-Luc; 3,  $p < 0.05$  for Ad-E2F-1 vs. control + RT; 4,  $p < 0.05$  for Ad-E2F-1 vs. Ad-Luc + RT; 5,  $p < 0.05$  for Ad-E2F-1 vs. Ad-E2F-1 + RT; 6,  $p < 0.05$  for Ad-E2F-1 + RT vs. control; 7,  $p < 0.05$  for Ad-E2F-1 + RT vs. Ad-Luc; 8,  $p < 0.05$  for Ad-E2F-1 + RT vs. control + RT; 9,  $p < 0.05$  for Ad-E2F-1 + RT vs. Ad-Luc + RT.

Table 2. Effect of Ad-E2F-1, Ad-p53, and Ad-Luc on LNCaP and PC3 plating efficiencies using analysis of variance

LNCaP	Ad-Luc/C*		Ad-E2F-1/C*		T-test†
10 MOI	0.68 ± 0.20		0.50 ± 0.06		0.200
25 MOI	0.33 ± 0.08		0.23 ± 0.07		0.159
50 MOI	0.29 ± 0.23		0.12 ± 0.04		0.166
PC3	Ad-Luc/C*	Ad-E2F-1/C*	Ad-P53/C*	(Ad-E2F1 + Ad-p53)/C*	Bonferroni†
10 MOI	0.85 ± 0.20	0.79 ± 0.36	0.11 ± 0.00	0.13 ± 0.14	2, 3, 4, 5
25 MOI	0.79 ± 0.14	0.48 ± 0.26	0.06 ± 0.04	0.09 ± 0.16	1, 2, 3, 4, 5
50 MOI	0.79 ± 0.17	0.14 ± 0.12	0.32 ± 0.23	0.00 ± 0.00	1, 2, 4

Abbreviations: Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1; C = Control; MOI = multiplicity of infection.

\* Plating efficiency for control was tabulated as mean percent survival of five experiments ± standard deviations (SD). The plating efficiency ratios were tabulated as ratios of the means ± SD of Ad-Luc or Ad-E2F-1 over means of the corresponding controls.

† Bonferroni test: 1,  $p < 0.05$  for Ad-E2F-1/C vs. Ad-Luc/C; 2,  $p < 0.05$  for Ad-p53/C vs. Ad-Luc/C; 3,  $p < 0.05$  for Ad-p53/C vs. Ad-E2F-1/C; 4,  $p < 0.05$  for (Ad-E2F-1 + Ad-p53)/C vs. Ad-Luc/C; 5,  $p < 0.05$  for (Ad-E2F-1 + Ad-p53)/C vs. Ad-E2F-1/C; 6,  $p < 0.05$  for (Ad-E2F-1 + Ad-p53)/C vs. Ad-p53/C.

The results of analysis of variance using the Bonferroni test to correct for multiple comparisons are shown. In LNCaP and PC3 cells, Ad-E2F-1 transduction did not significantly enhance Annexin V levels. When combined with RT, Ad-E2F-1 exposure resulted in a significant increase (at least additive) in Annexin V staining in LNCaP cells, but not PC3 cells. In contrast, Ad-E2F-1 alone was sufficient to significantly increase caspase 3 + 7 activity in LNCaP cells. The addition of RT to Ad-E2F-1 transduced LNCaP cells did not significantly enhance caspase 3 + 7 levels compared with

Ad-E2F-1 alone. In the p53<sup>null</sup> PC3 cell line, Ad-E2F-1 alone did not significantly enhance caspase 3 + 7 activity over that of Ad-Luc, but did result in significantly increased activity when combined with RT.

Clonogenic survival plating efficiencies of LNCaP and PC3 cells are listed in Table 2. Each experiment was repeated five times, and the mean and standard deviations were calculated for each set of experiments. To adjust for variations in experimental conditions, the plating efficiencies of cells transduced with Ad-Luc, Ad-E2F-1, Ad-p53,

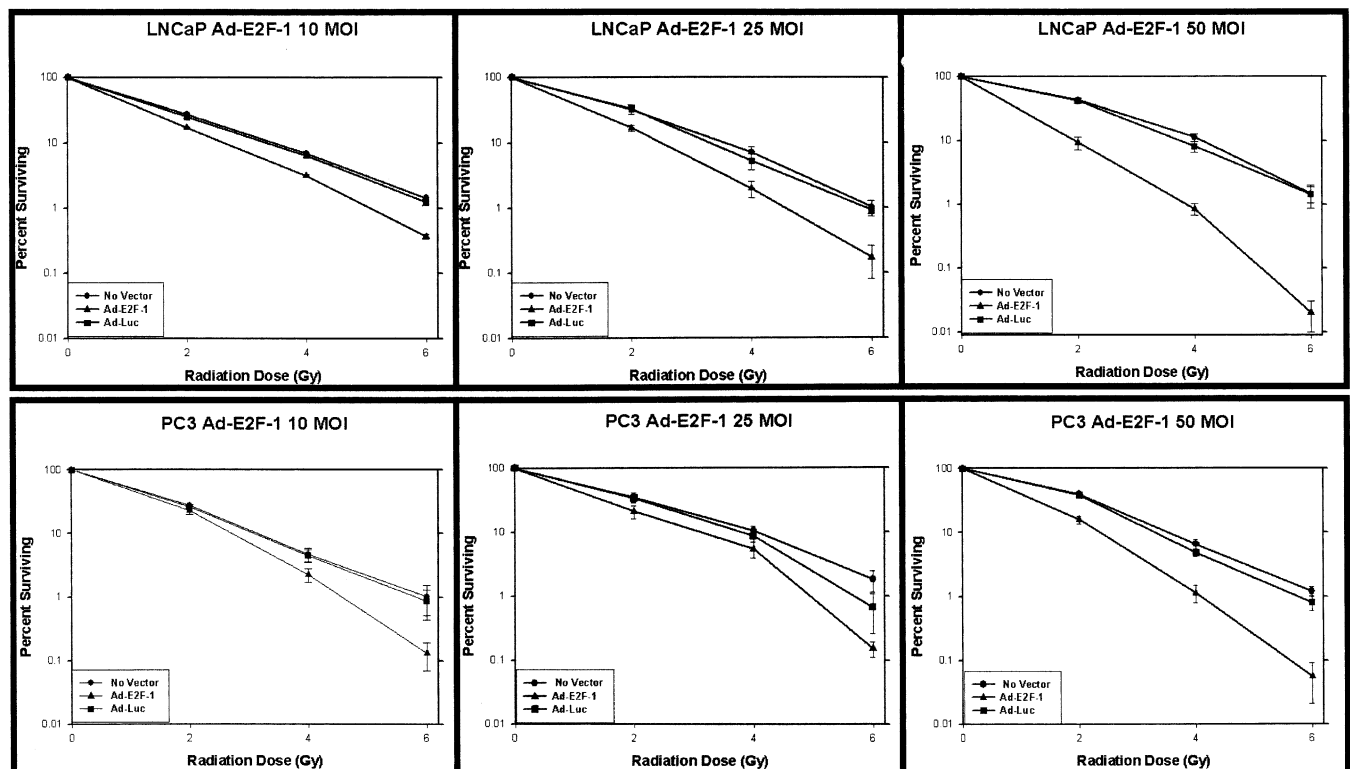


Fig. 3. Effect of Adenoviral-E2F-1 (Ad-E2F-1) on clonogenic survival of p53<sup>wild-type</sup> (LNCaP) and PC3 cells. LNCaP and PC3 cells were transduced with Ad-E2F-1 at multiplicity of infection of 10, 25, and 50.

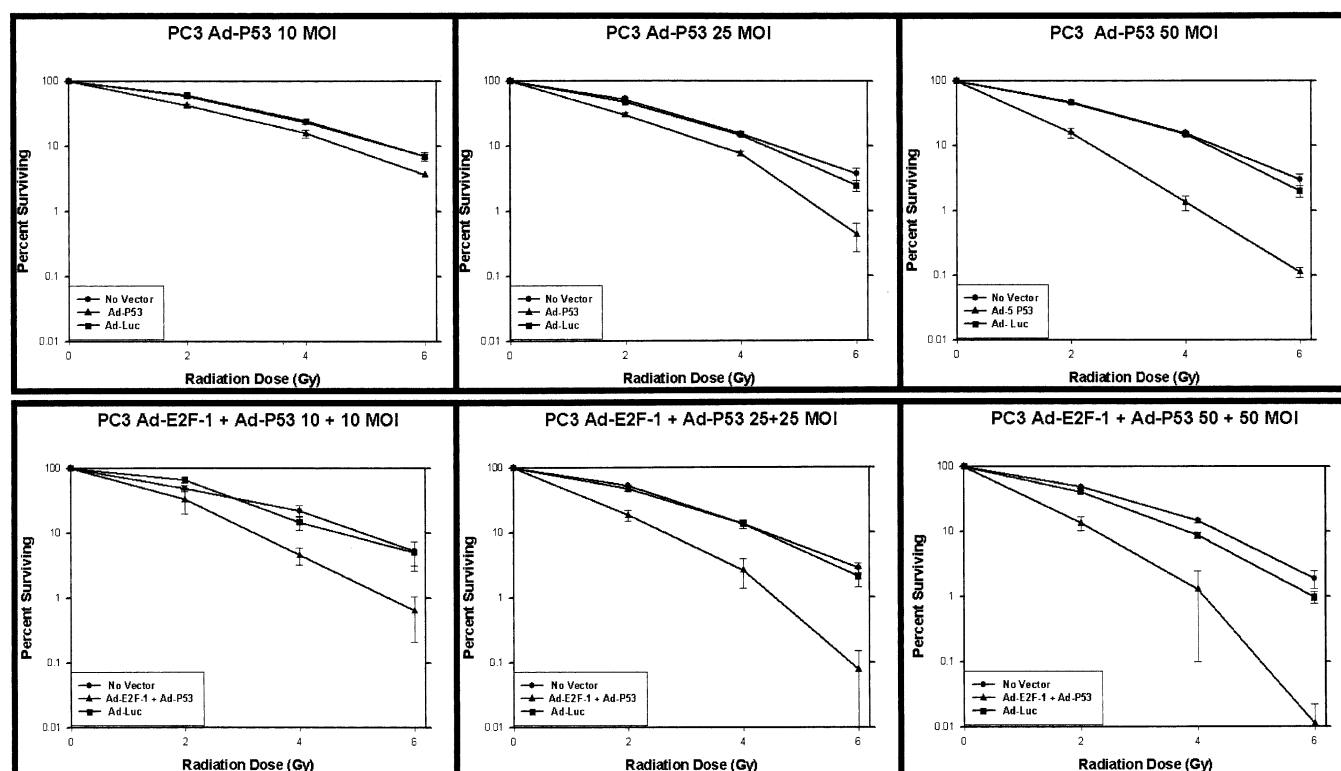


Fig. 4. Effect of Ad-p53 and 1 Ad-E2F-1 + Ad-p53 on clonogenic survival of PC3 cells. PC3 cells were transduced with Ad-p53 alone or in combination with Ad-E2F-1 at multiplicity of infection (MOI) of 10, 25, and 50. The Adenoviral-Luciferase control was administered at MOIs of 10, 25, and 50.

and the combination of Ad-E2F-1 + Ad-p53 were each normalized to their respective controls and expressed as ratios of the mean. For LNCaP and PC3 cells incubated in control medium, the mean ( $\pm$ SD) plating efficiencies were 15.5% ( $\pm$ 5.6%) and 37.7% ( $\pm$ 17.7%), respectively. In general, increasing the MOI from 10 to 50 reduced the plating efficiency for all of the viral vectors used. One exception was for PC3 cells treated with Ad-p53 alone, in which there was no statistically significant difference in the plating efficiencies at 10, 25, or 50 MOI. Ad-p53 alone or in combination with Ad-E2F-1 was effective in reducing PC3 cell plating efficiency relative to Ad-Luc.

In LNCaP cells, the addition of Ad-E2F-1 did not significantly affect plating efficiency relative to Ad-Luc. However, Ad-E2F-1 significantly reduced PC3 cell survival compared with Ad-Luc at MOIs of 25 and 50.

The effects of E2F-1 on cell survival normalized to plating efficiency in the absence of RT are shown in Fig. 3 and Fig. 4. Ad-E2F-1 significantly radiosensitized LNCaP and PC3 cells. In LNCaP cells, radiosensitization was observed at all RT dose levels when Ad-E2F-1 at MOI of 10 was added (Table 3). In the PC3 cell line, significant radiosensitization by Ad-E2F-1 required an MOI of 50; neither an Ad-E2F-1 MOI of 10 nor 25 was sufficient to produce significant differences compared with controls (Table 4). Exposure of p53<sup>null</sup> PC3 cells to Ad-p53 at an MOI of at least 25 also significantly increased the radiation response

of PC3 cells (Table 5). When p53 replacement with Ad-p53 was combined with Ad-E2F-1 there was at least an additive effect on PC3 cell radiosensitization that was most evident at an MOI of 25 for each vector (Table 6, Fig. 4).

## DISCUSSION

Prostate cancer remains a leading killer of men. Although widespread use of prostate-specific antigen and digital rectal exam screenings have led to earlier diagnosis, a significant number of men still present with high-risk clinically localized disease (35, 36). About half of such men will experience recurrence after definitive therapy. Local recurrence remains a major cause of failure despite improvements in radiation treatment delivery, radiation dose escalation, and combined treatment with androgen deprivation (37–39). Targeted biologic therapy holds promise for improving radiation response.

In this study, we demonstrated the efficacy of Ad-E2F-1 in enhancing the radiosensitivity of two prostate cancer cell lines using apoptotic and clonogenic survival assays. Annexin V and caspase 3 + 7 assays, used to estimate cell death, showed that Ad-E2F-1 transduction increased apoptosis. Although Ad-E2F-1 enhanced Annexin V and caspase 3 + 7 activities in both cell lines, these effects were more significant in LNCaP cells. Using clonogenic survival assays, we demonstrated that Ad-E2F-1 transduction sub-

Table 3. Comparison of clonogenic percent survival of Ad-E2F-1 transduced LNCaP cells using analysis of variance

10 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	27.16 ± 1.02	24.79 ± 1.59	17.20 ± 0.72	1, 2
4 Gy	6.86 ± 0.34	6.43 ± 0.65	3.15 ± 0.22	1, 2
6 Gy	1.43 ± 0.10	1.23 ± 0.24	0.37 ± 0.05	1, 2
25 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	31.76 ± 8.28	32.53 ± 9.10	16.84 ± 3.39	NS
4 Gy	7.04 ± 2.87	5.24 ± 2.48	1.97 ± 0.95	NS
6 Gy	1.01 ± 0.47	0.90 ± 0.17	0.17 ± 0.15	1
50 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	43.00 ± 5.17	41.48 ± 5.68	9.26 ± 4.63	1, 2
4 Gy	11.20 ± 3.63	8.05 ± 3.55	0.85 ± 0.38	1, 2
6 Gy	1.46 ± 0.89	1.43 ± 1.25	0.02 ± 0.02	NS

*Abbreviations:* Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1; MOI = multiplicity of infection; NS = Nonsignificant; R = radiation therapy.

\* Clonogenic percent survival is tabulated as mean of 5 experiments ± standard deviations.

† Bonferroni test: 1,  $p < 0.05$  for Ad-E2F-1 vs. control; 2,  $p < 0.05$  for Ad-E2F-1 vs. Ad-Luc; NS,  $p > 0.05$ .

stantially increased overall cell death in p53<sup>wild-type</sup> LNCaP cells and p53<sup>null</sup> PC3 cells. Even though p53 was not required for radiosensitization from E2F-1 overexpression,

higher titers of Ad-E2F-1 were required to produce similar decrements in cell survival in PC3 cells. This difference in radiosensitization from Ad-E2F-1 between LNCaP and PC3

Table 4. Comparison of clonogenic percent survival of Ad-E2F-1 transduced PC3 cells using analysis of variance

10 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	28.02 ± 1.76	26.10 ± 1.76	23.03 ± 6.66	NS
4 Gy	4.70 ± 2.60	4.50 ± 2.36	2.24 ± 1.22	NS
6 Gy	1.01 ± 1.12	0.87 ± 0.98	0.13 ± 0.14	NS
25 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	31.76 ± 8.28	32.53 ± 9.10	16.84 ± 3.39	NS
4 Gy	10.31 ± 4.74	8.65 ± 3.81	5.04 ± 3.38	NS
6 Gy	1.79 ± 1.48	0.67 ± 0.90	0.11 ± 0.12	NS
50 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1*	Bonferroni test†
2 Gy	39.74 ± 2.61	38.43 ± 5.66	15.98 ± 5.20	1, 2
4 Gy	6.60 ± 2.67	4.82 ± 1.61	1.13 ± 0.84	1, 2
6 Gy	1.20 ± 0.49	0.80 ± 0.54	0.06 ± 0.07	1, 2

*Abbreviations:* Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1; MOI = multiplicity of infection; NS = nonsignificant; RT = radiation therapy.

\* Clonogenic percent survival is tabulated as mean of five experiments ± standard deviations.

† Bonferroni test: 1,  $p < 0.05$  for Ad-E2F-1 vs. control; 2,  $p < 0.05$  for Ad-E2F-1 vs. Ad-Luc; NS,  $p > 0.05$ .



Table 5. Comparison of clonogenic percent survival of Ad-p53 transduced PC3 cells using analysis of variance

10 MOI				
RT dose	Control*	Ad-Luc*	Ad-p53*	Bonferroni test†
2 Gy	58.13 ± 2.34	60.30 ± 5.90	41.89 ± 4.24	NS
4 Gy	4.70 ± 2.60	4.50 ± 2.36	2.24 ± 1.22	NS
6 Gy	6.97 ± 0.69	6.89 ± 1.47	3.58 ± 0.00	NS
25 MOI				
RT dose	Control*	Ad-Luc*	Ad-p53*	Bonferroni test†
2 Gy	51.75 ± 5.25	46.98 ± 1.01	29.87 ± 3.34	1, 2
4 Gy	15.41 ± 1.79	14.60 ± 0.65	7.45 ± 1.09	1, 2
6 Gy	3.66 ± 1.33	2.44 ± 0.76	0.43 ± 0.35	1
50 MOI				
RT dose	Control*	Ad-Luc*	Ad-p53*	Bonferroni test†
2 Gy	46.85 ± 4.25	45.91 ± 1.27	15.73 ± 5.55	1, 2
4 Gy	15.48 ± 2.02	14.91 ± 1.56	1.33 ± 0.66	1, 2
6 Gy	2.96 ± 1.19	1.98 ± 0.81	0.11 ± 0.04	1, 2

*Abbreviations:* Ad-Luc = Adenoviral-Luciferase; Ad-p53 = Adenoviral-p53; MOI = multiplicity of infection; NS = nonsignificant; RT = radiation therapy.

\* Clonogenic percent survival is tabulated as mean of five experiments ± standard deviation.

† Bonferroni test: 1,  $p < 0.05$  for Ad-p53 vs. control; 2,  $p < 0.05$  for Ad-p53 vs. Ad-Luc; NS,  $p > 0.05$ .

cells may be due in part to other downstream factors and not necessarily on codependence of E2F-1 and p53. Nevertheless, when PC3 cells were cotransduced with Ad-E2F-1 and

Ad-p53, there was at least an additive increase in radiation induced overall cell death.

Our results in prostate cancer cell lines are in keeping

Table 6. Comparison of clonogenic percent survival of Ad-E2F-1 and Ad-p53 transduced PC3 cells using analysis of variance

10 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1 + Ad-p53*	Bonferroni test†
2 Gy	48.61 ± 10.28	65.45 ± 12.92	32.62 ± 26.13	NS
4 Gy	21.92 ± 8.90	14.64 ± 6.99	4.54 ± 2.73	1
6 Gy	5.20 ± 4.25	5.00 ± 4.15	0.63 ± 0.84	NS
25 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1 + Ad-p53*	Bonferroni test†
2 Gy	52.98 ± 1.88	46.85 ± 6.15	18.33 ± 6.33	1, 2
4 Gy	13.45 ± 3.53	13.43 ± 0.36	2.61 ± 2.13	1, 2
6 Gy	2.77 ± 0.87	2.05 ± 1.15	0.12 ± 0.16	NS
50 MOI				
RT dose	Control*	Ad-Luc*	Ad-E2F-1 + Ad-p53*	Bonferroni test†
2 Gy	47.99 ± 2.99	49.68 ± 5.26	13.26 ± 4.45	1, 2
4 Gy	14.31 ± 1.19	13.31 ± 0.41	1.28 ± 1.67	1, 2
6 Gy	N/A	N/A	N/A	N/A

*Abbreviations:* Ad-Luc = Adenoviral-Luciferase; Ad-E2F-1 = Adenoviral-E2F-1; Ad-p53 = Adenoviral-p53; MOI = multiplicity of infection; NS = nonsignificant; N/A = not available; RT = radiation therapy.

\* Clonogenic percent survival is tabulated as mean of five experiments ± standard deviation.

† Bonferroni test: 1,  $p < 0.05$  for Ad-E2F-1 + Ad-p53 vs. control; 2,  $p < 0.05$  for Ad-E2F-1 + Ad-p53 vs. Ad-Luc; NS,  $p > 0.05$ .

with other investigations involving different cell lines. In serum-starved fibroblasts, Qin and colleagues (40) demonstrated that E2F-1 overexpression led to p53-dependent apoptosis. Bargou *et al.* (41) showed that inhibition of E2F-1 in a normal breast epithelial cell line inhibited apoptosis and induced tumor growth in SCID mice. Similarly, Shan and Lee (9) confirmed that REF52 and RAT2 cell lines lost their ability to undergo apoptosis when E2F-1 was mutated. A potential mechanism forwarded by Kowalik and colleagues (42) was that E2F-1 promotes p53 dependent apoptosis by sequestering MDM2 to prevent ubiquitination and degradation of p53. As in our study, they showed that overexpression of E2F-1 led to accumulation of p53. Studies by Hsieh *et al.* (43) and Kowalik *et al.* (42) independently confirmed that overexpression of MDM2 limited native E2F-1's ability to induce p53-dependent apoptosis.

p53 may play a key role in E2F-1-mediated apoptosis; however, other studies have suggested a p53-independent mechanism similar to our results with p53<sup>null</sup> PC3 cell line. Pruschy and colleagues (10) demonstrated that over-

expression of E2F-1 increased radiation sensitivity in a p53-negative fibrosarcoma cell line. Macleod *et al.* (44) showed that unregulated E2F-1 activity in a mouse peripheral nervous system lacking functional pRb led to increased apoptosis independent of p53 status. A testicular tumor model studied by Holmberg *et al.* (45) also suggested that the apoptotic cascade was intact regardless of p53 status.

In conclusion, we have shown for the first time in human prostate cancer cell lines that Ad-E2F-1 is a potent radiosensitizer, particularly when wild-type p53 is present. Because most early prostate cancers express functional p53, Ad-E2F-1 should have considerable activity. p53 mutations are much more prevalent in locally advanced cancers and our results suggest at least an additive radiosensitizing effect of Ad-p53 and Ad-E2F-1 in prostate cancer cells lacking functional p53. In this setting, p53 replacement with Ad-p53 might be a useful adjunct to Ad-E2F-1 and RT. These findings suggest a potential role of Ad-E2F-1 gene therapy in the radiotherapeutic management of prostate cancer.

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